CHROMIDIAL EXTRUSION, AND ITS RELATIONSHIP TO ATYPICAL NUCLEAR PHENOMENA IN TUMOUR CELLS[1]

by

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(Submitted for publication 8th August, 1930.)

INTRODUCTION.

When R. Hertwig (5) formulated the so-called "chromidial hypothesis" in 1902, the importance of this phenomenon was at once realized, as any interchange between the nucleus and the cytoplasm must necessarily be one of the most fundamental of all living cellular processes.

Shortly afterwards the findings of Hertwig were confirmed by the observations of Wassilieff (13), Popoff (9), and Goldschmidt (4), and in 1909 Dobell (2) expanded the chromidial theory when he published his critical review entitled "Chromidia and the bi-nuclearity hypothesis."

Since then many conflicting theories have been brought forward which suggested the rôle played by these cytoplasmic organs during metabolism, and more particularly during the cellular differentiation of embryonic tissues.

The chromidial theory first originated in protozoology, and the formation and subsequent behaviour of this extra-nuclear material has been studied extensively within the protista, protozoa, and the lower metazoa.

Up to the present time very little cytological work has been carried out upon the cells of the higher vertebrates. More recently, however, the presence of the chromidial apparatus has been reported within the cells of sarcomatous new growths by Ludford, Richardson, and Horning.

Since the chromidial material is the vehicle of interchange between the nuclearplasm and the general protoplasm of the living cell during intermitosis, it is therefore assumed that this extruded basophilic substance is an important factor in cell physiology. In the hope of seeking some new cytological principle or criterion for malignancy, and in order to help to elucidate the functional significance of the chromidial complex, detailed cytological studies have been extended to those cells composing neoplasms.

[1]This work was carried out with the aid of the Cancer Research and Treatment Fund, New South Wales.

MATERIALS AND METHODS. CLINICAL OBSERVATIONS.

The following studies are based upon material obtained from human and rodent sarcomata. In all cases the tissues were fixed immediately after death, which is imperative in order to obtain precise cytological results. The rodent material selected was a round-celled, transplantable mouse sarcoma, which has been successfully cultivated in this laboratory during the past two years.

The latter was fixed in Benda's modified Flemming's solution, and the human growth was treated with Benda's alizarin technique, Regaud's formol bichromate, or else in a strong Flemming's solution, without acetic acid. Both kinds of neoplastic tissue were subsequently stained with Heidenhain's iron haematoxylin and occasionally counter-stained with eosin.

The human new growth was obtained from a woman aged forty. Ten weeks prior to her death the patient had observed an ulcerated swelling in the soft palate, which had progressed very rapidly in size. Seven weeks after the tumour was first noticed it was submitted to a dose of deep ray-therapy. Within two days of this treatment the tumour had visibly decreased in size, but in a further three days the supra-clavicular glands became very much enlarged, and two days later the axillary glands were observed to increase in size, and then develop into a considerable mass. From this time until her death (a period of two weeks) tumours appeared with alarming rapidity all over her body. Many metastases grew over night, and attained a considerable size. Three weeks after the commencement of treatment the patient died, with evidence of widespread metastases in the viscera and other portions of the body. No autopsy was permitted, but the neoplasm was diagnosed pathologically as a mixed-celled sarcoma.

HISTORICAL RESUME.

Before Hertwig formulated the chromidial hypothesis, he succeeded in 1899 in observing the formation of secondary nuclei from the chromidial mass present in the cytosome of certain of the lower organisms, and subsequently Elpatiewsky (3) demonstrated that the macro- and micro-amoebae, into whose formation the chromidial nuclei or secondary nuclei enter, are gametes which conjugate in pairs. Still later these observations were followed up in great detail by Swarczewsky (10), who showed that "chromidiogamy" may occur in which the two chromidial masses of both cells undergo complete conjugation. In 1902 Schaudinn (11) described a process of chromidiosis within the macrogametocyte of the malarial parasite, and Hertwig interpreted this extrusion as a process which balances the karyoplasmic relations of the cell.

Goldschmidt was among the first workers to put forward the idea that chromidia play an important rôle in cellular differentiation, and he even went

so far as to suggest that these cytoplasmic organs actually differentiate into various functional structures; while Schazel (12) contended that chromidiosis plays the rôle of converting the undifferentiated cells into the specialized cells of the somatic tissues. His evidence for this phenomenon appears to be based mainly upon the fact that during early embryonic development of the embryo a chromidial substance cannot be detected until actual differentiation of the tissues occurs.

Further observations upon early cell development led Hertwig to suggest that chromidia in mammals may transform into yolk, and more recent investigators support the idea that the deutoplasmic granules arise from the chromatic material extruded from the nuclear mechanism.

Some modern cytologists conclude that the cell nucleus, which is the centre of cellular metabolism, directs and controls the general protoplasmic activities by means of chromidial extrusion into the cytosome.

With the improvements in the cytological technique, the conclusions of these earlier works gradually became discredited, and once again the chromidial complex has entered into the arena of investigation, and, realizing the fundamental rôle this extra-nuclear material must necessarily play in cell phenomena, the field is promising.

MANNER OF NUCLEAR EXTRUSION.

The chromidium, unlike the mitochondrium, is apparently not a permanent cell-organ specific to all cell-types, but is rather to be regarded as a physiological expression of active cellular metabolism. Also, unlike the latter type of cytoplasmic inclusion, the chromidial complex possesses no definite morphological individuality, and invariably presents itself in the form of an irregular mass with the cytosome of the cell.

It is generally contended by most cytologists that two distinct kinds of chromatin exist: nutritive trophochromatin and generative or somatic idiochromatin, the former being derived from the latter. As to whether this apparent differentiation of chromatic material takes place only within the nuclearplasm during intermitosis, and then passes through the membrane in the two forms, or whether this process may also occur after this extra-nuclear substance has reached the cytosome, is a matter of speculation. The manner in which chromatic material becomes extruded through an intact membrane is uncertain, and still more interesting is this phenomenon in view of the recent work of Chambers (1), who has demonstrated that the nuclear wall is a true morphological membrane, and can be thrown into wrinkles when the nucleus is compressed.

It certainly appears unlikely that formed bodies would be permitted to pass through, and one would naturally speculate that the chromatic material

would pass through in a liquid form with substances in solution, and therefore the substances would be composed of relatively small molecules, which would be able to pass through the nuclear wall by exosmosis.

A few cytologists, among whom is Hertwig, maintain that the nuclear substance becomes extruded into the cell-protoplasm by means of breaks in the nuclear wall, and this author, while observing chromidiosis in *Actinosphaerium*, contends that the nuclear membrane breaks down, thereby liberating the nuclear material into the naked cytoplasm.

In certain infusorians one of us has depicted a somewhat similar phenomenon, in which apparent karyolysis is followed by chromidial reorganization into a secondary nucleus from the residual chromidia present in the cytosome, which resembles the endomictic process previously described by Woodruff and Erdmann (14) in the other forms of the protozoa infusoria.

As endomixis or natural parthenogenesis does not play a fundamental economic rôle in the more highly organized somatic cell, and bearing in mind the physiological differences in cellular activity existing between the protozoan and the somatic cell of the higher vertebrates, one must therefore look for a difference in the functional significance of chromidial behaviour between the two cell-types.

It might even be suggested that the chromidia may arise from the residual chromatin, which lies naked in the cytoplasm after the nuclear membrane becomes dissolved during the late pro-phase, but the evidence obtained during this investigation shows that this chromatic material is capable of passing through the intact nuclear wall by means of exosmosis during inter-kineses (see figure 5), and apparently becomes reconstituted on reaching the cytosome.

This manner of extrusion is in accord with the previous observations of Horning and Richardson (6) during chromidiosis in sarcoma cells, the functional significance of which will be discussed in a later portion of this communication.

CHEMICAL COMPOSITION OF THE NUCLEARPLASM.

Owing to the great difficulty in the past of obtaining chromatin separate from other constituents of the cell, very little is, unfortunately, known concerning the chemical composition of the nucleus. Investigators, however, have succeeded in examining chromatin separately, and this material has now been obtained from the nuclearplasm of the thymus, as well as from the cells of the spermatozoa of echinoderms.

With the aid of the micro-pipette it has been found possible to extract the nuclearplasm from the various cells, and by employing this method it was discovered that nucleic acid is a constituent of the chromatin, and, moreover, that chromatin consists of a salt of nucleic acid with a protein base.

As the form and function of cells vary, even within the same animal species, one can only generalize; nevertheless, the amount of nucleic acid is found to vary only slightly in different cell-types, and, according to Mathews (8), the protein base was found to be more or less constant in most cells of different types. Taking into consideration the general functional differences existing between animal and plant cells, the nucleic acid of their nucleus is found to be on the whole more or less uniform.

Further work has been carried out upon the nucleo-proteins in fish spermatozoa, which shows that the protein in question is simple and belongs to the group of protamines. Experiments upon the chromatin of the erythrocytes of birds has demonstrated that a histone is involved, which is a simple basic protein containing more kinds of amino-acids in its molecule than that of the protamines. Mathews further records that in the spermatozoa of certain sea-urchins the basic component is a substance related to both protamines and histones.

The cytological evidence concerning the two kinds of chromatin has been reported by the chemical observations of Kössel (7), who assumes that the differences between the two kinds of chromatic material are dependent upon their basic or protamine compounds, and not upon their nucleic acid.

At the present moment there is no evidence which suggests that this extra-nuclear substance alters its chemical nature after it has reached the cytosome from the nuclearplasm of the living cell.

CHROMIDIOSIS AND ITS RELATIONSHIP TO NUCLEAR PHENOMENA.

Cytological Differences between Stromal and Malignant Cells.

The sarcomata differ from other forms of neoplastic growths inasmuch as they are composed of highly differentiated cell-types. Following the cytological treatment already described, a sharp cytological differentiation of the neoplastic component from the stromal element gives at once a diagnostic character to this type of tumour which is not common to the carcinomata.

This cytological alteration of the malignant cell-elements of the sarcomata is replaced in epithelial growths by a process involving a gradual cell transformation, which invariably produces no other cytological feature than that of hyperchromasy and plastin hypertrophy.

An examination of the microphotograph (see figure 1), showing the invading malignant tissue and the adjoining fibrous stroma, reveals various cytological differences between the neoplastic and the normal cell. Tumour cells composing the sarcomatous tissues resemble mature lymphocytes, the majority of which are oval or rounded in shape, and occasionally more elongated types may be detected. The large hyperchromatic nucleus occupies nearly the whole

of the cell-body, and the general protoplasm forms only a thin layer around the periphery of the nucleus. The cytoplasm, which is generally recognized by its reticular structure, shows more selective affinity for the stain than do those cells forming the stromal tissue.

The whole of the general cytosome presents a somewhat granular aspect, probably due to an abnormal increase of cytomicrosomes and the products of greater metabolic activity. The most conspicuous cytological feature of the tumour cell is that of nuclear hyperchromasy, and it affords greater contrast when compared with the transparent normal nuclei of the stromal cells (see figure 1).

Chromidial Extrusion.

The process of chromidiosis, in those cells composing the malignant tissues of a rodent sarcoma, has recently been depicted by Horning and Richardson in a previous communication (6), in which it was shown that the typical hyperchromatic tumour cells are capable of extruding chromidia, which normally remain within the cytosome, through the wall of the cell, where, on reaching the surrounding matrix, the material apparently becomes dissolved.

The authors interpret this phenomenon in view of the hypothesis of Popoff (9), who contends that this is a process by which the mass relation between nucleus and cytoplasm may be restored if, for any reason, the amount of chromatin relative to the protoplasm has increased.

It was also revealed that chromidiosis was invariably associated with those neoplastic cells undergoing unequal amitotic division (see figure 2). Previous observations of these authors have shown that asymmetrical division is a phenomenon rarely met with within the healthy normal cell, but, nevertheless, may be encountered during the early phases of cytolysis *in vitro*, and it was contended that the degenerating cell liberates into the culture medium a substance which incurs asymmetrical division, and the possibility that a similar substance may be set free from the malignant cell during autolysis of the necrotic elements was also suggested.

Previous workers are of the opinion that this abnormality, readily depicted in cancer cells, is brought about by some inherent nuclear mechanism, and our contention that this phenomenon may be induced by some extrinsic chemical principle is of interest.

Extensive cytological examination of both types of human and rodent sarcomata shows clearly that this abnormal nuclear behaviour is confined to those cells comprising the neoplastic tissues, and could not be detected within the stromal elements adjoining the new growth.

Brailsford Robertson assumed that asymmetrical division plays an important rôle in the causation of cancer, and concludes that, following unequal division, the daughter cells containing the larger nucleo-cytoplasmic ratio are

unable to multiply, and are gradually overwhelmed by the rapidly increasing tumour cells, which arise from those elements possessing the lower nucleo-cytoplasmic ratio.

Our previous studies upon normal cells undergoing a symmetrical amitosis during the early phases of cytolysis *in vitro,* indicated that the daughter portion containing the smaller amount of nuclear and protoplasmic substance migrates independently within the culture medium, and apparently, owing to intracellular tension forces, disintegrates and ultimately disappears. Thus after the karyoplasmic mechanism becomes absorbed, the cell containing the larger nucleo-cytoplasmic ratio survives, and, as far as can be ascertained, leads an independent normal existence.

Examinations of mixed cell metastatic deposits depict the chromidial apparatus in all stages of extrusion, from the nuclearplasm until its final emission to the cell exterior. Frequently large droplets of chromidia are seen adhering to the outer surface of the cell membrane, where they remain prior to their final disintegration within the matrix (see figures 3, 4). The photomicrograph (figure 5) depicts the chromidial substance actually in the process of passing through what appears to be an intact morphological nuclear membrane, and in view of the controversy concerning the manner in which this material reaches the cytosome this behaviour is of special interest.

A systematic study of chromidiosis within the malignant components of various sarcomata suggests that the more malignant the growth the more active and intense is the chromidial emission.

This nuclear process has been so active in the abnormally growing mixed-cell growth that some of the cells show nuclear depletion of chromatin (see figures 4, 5), and the general field presents a somewhat confused appearance, owing to large masses of chromidia, which are seen either embedded in the surrounding ground substance or else adhering to the periphery of the cell-walls of the malignant elements.

The explantation of malignant growths *in vitro* has demonstrated that the neoplastic elements liberate into the culture medium a growth-promoting principle, which brings about active cellular proliferation of normal healthy tissues, and bearing in mind the chemical nature of chromidia it might be suggested that this growth-stimulating principle may be correlated with the emission of chromidial substance during chromidiosis in active growing tumours.

Microscopic observations of a mixed-cell deposit reveal in certain areas the presence of multi-nucleated cells, whose nuclei vary from two to ten in number. Taking into consideration the general morphology and cytological staining reactions of these multi-nucleated components, the possibility that they are derived from the malignant elements, and not from the stromal components, as has been previously contended, is highly suggestive.

The presence of large chromidial masses, lying within the narrow protoplasmic spaces between the nuclei, is another typical cytological feature of this cell-type. Extrusion of this material to the exterior of the cell-wall appears to be a constant feature, but observations suggest that the chromidia lying within the cytosome are capable of penetrating the membranous wall of the adjacent nuclei (see figure 6).

A critical study of the normal unicellular elements shows that nuclear depletion, owing to abnormal chromidial emission, is a factor invariably associated with the process of invagination of the nuclear walls of those cells composing the rapidly growing new growth. *We therefore assume that the withdrawal of chromatin from the cell-nucleus upsets the tension forces, which normally exist, between the cytosome and nuclearplasm, thereby causing the nuclear membrane to become indented and constricted, giving all stages up to the complete constriction of the achromatic nucleus into two closely approximate halves. It is further contended that this phenomenon not only restores the karyoplasmic relations of the cell, but is the process from which the multi-nucleated condition is derived.*

SUMMARY.

1. Although chromidiosis may be associated with conjugation and other reproductive phenomena in protozoa, it is correlated with different processes in cells of the higher metazoa.

2. Evidence is given for the assumption that chromidia in the latter cells pass through the nuclear membrane in solution by exosmosis.

3. Chromidiosis has been described in sarcoma cells composing human and rodent new growths, and its activity appears to vary directly with the metabolism and growth rate of the neoplasm.

4. It is suggested that chromidia take part in the liberation of growth-promoting substances found in *in vitro* cultures of malignant tissues.

5. The withdrawal of chromatin from the nucleus upsets the tension forces existing between the cytosome and the nuclearplasm, and leads to invaginations and constrictions in the nuclear membrane.

6. The multi-nucleated condition common in neoplasms and healthy cells undergoing cytolysis results from repeated nuclear constriction following chromidial emission.

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EXPLANATION OF PLATES.

Figure 1. Photomicrograph of a fixed section of a round-celled mouse sarcoma after treatment with Regaud's formal bichromate and stained with Heidenhain's ferric haematoxylin. Observe the differences in staining reaction between the darkly stained invading sarcomatous tissues and the healthy fibrous stroma. Note especially the hyperchromasy of the malignant cells, together with deeply staining cytoplasm. Contrast this with the normal chromatic structure of stromal cells.

Figure 2. Depicting a small round-celled mouse sarcoma that has been previously fixed with a modified Benda's solution, and stained with Heidenhain's iron alum. Note the morphology of the highly differentiated hyperchromatic sarcoma cells. Several of these cells in the centre of the field contain small chromidial bodies aggregated in the peripheral protoplasm prior to extrusion into the surrounding matrix. Cells marked 1, 2, and 3 are undergoing asymmetrical division. X, emission of chromidial material.

Figure 3. Photomicrograph of section of human mixed-celled sarcoma, showing malignant cells. N. depicts nucleus, C. intra-nuclear chromatin, Ch, chromidia aggregated in a large body within the peripheral cytoplasm prior to extrusion into the matrix. This tissue has been treated with the Benda alizarin technique.

Figure 4. The same. Observe hypochromatic nucleus owing to chromidial emission. Ch. depicts large chromidial masses, some of which are adhering to and imbedded within the peripheral protoplasm of the cells. Other masses are seen which have migrated into the matrix. Cyt. shows cytoplasmic region, N. the nucleus, Con. invagination of the nuclear membrane.

Figure 5. The same. Ch. depicts large irregular chromidial body photographed when passing through the nuclear membrane. Observe depletion and Con., invagination of nuclear membrane following chromidial emission. Lettering the same as in Figure 4.

Figure 6. The photomicrograph depicts the large multinucleated condition of a malignant cell marked M. Seven nuclei may be seen. Ch. shows a large mass of chromidial substance in the process of migrating from the nuclear membrane to the adjacent nucleus. Note also granular structure of protoplasm.

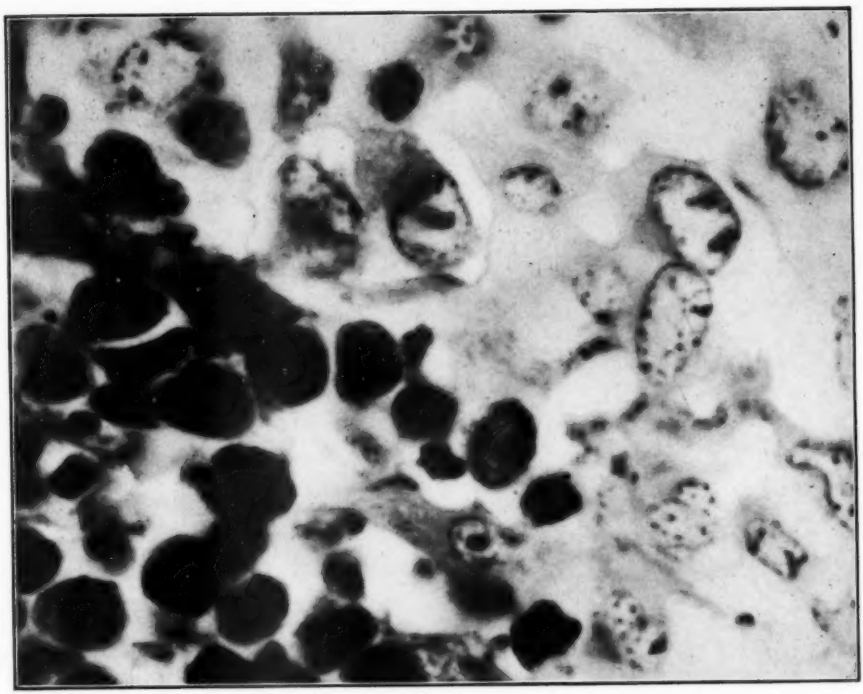
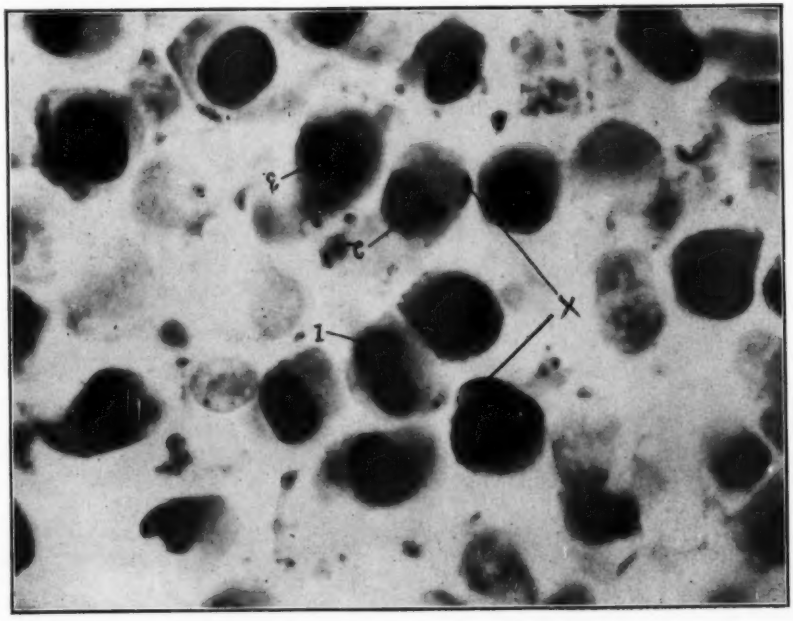


Fig. 1



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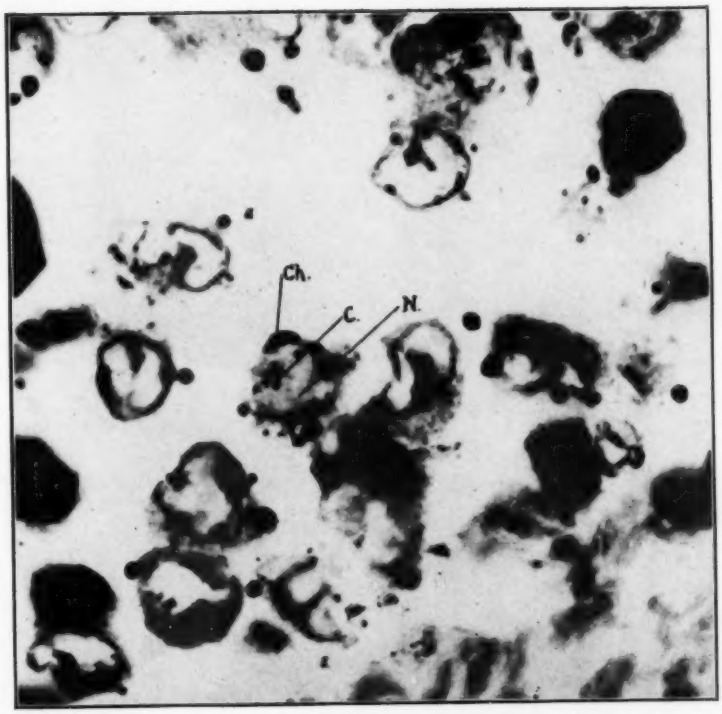
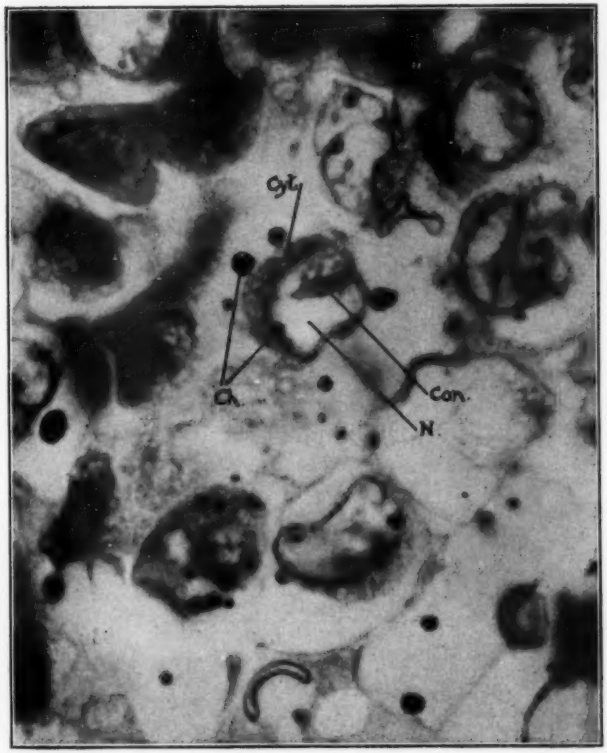


Fig. 3.



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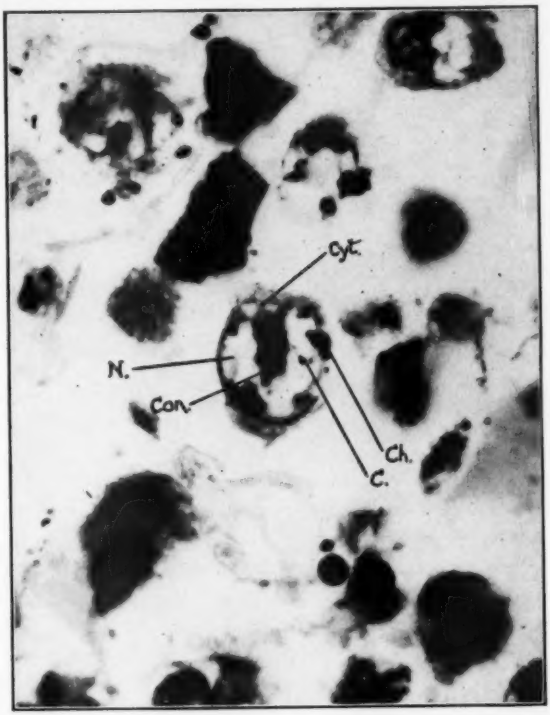

Fig. 5

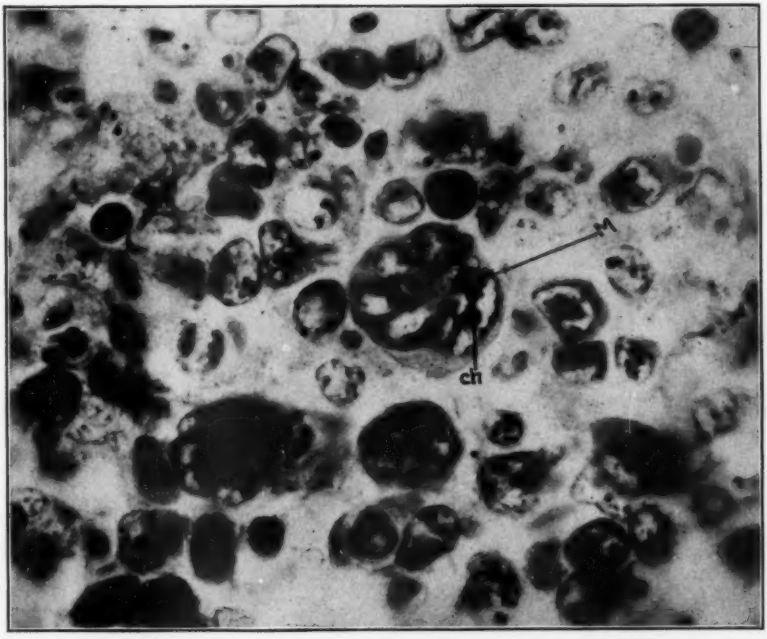

Fig. 6

THE MICRO-ORGANISMS OF THE TERTIARY RED SANDS NEAR MELBOURNE, VICTORIA

by

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(Submitted for publication 2nd October, 1930.)

INTRODUCTION.

In a previous paper (2) an account was given of the monthly variations in the numbers and the types of soil micro-organisms. The results were obtained by taking samples of soil at about monthly intervals during the year 1927 from a clay loam in the Silurian area near Melbourne.

It was found that there were certain fluctuations in the numbers of each type of organism, and that the variations corresponded to a certain extent with the fluctuations in soil moisture and temperature.

During the years 1928 and 1929 samples of soil were taken in the Tertiary red sands area which lies to the north-east of Port Phillip Bay. It was thought that the results would make an interesting comparison with those obtained the previous year.

AIM.

The main object of the investigation was to determine the following points:

1. Whether the sandy area showed the same fluctuations in numbers of micro-organisms with relation to the soil moisture and temperature as had been observed in the previous year.
2. Whether both soil areas supported the same numbers of each type of micro-organism.
3. Whether the same differences were observed between cultivated and virgin soil in both areas.
4. Which genera were common to both regions.

METHOD.

The area chosen for sampling was in the Cheltenham district, near Melbourne. The virgin bush soil was obtained from a reserve which has been preserved in its natural state. The vegetation consists of xerophilous scrub typical

of the coastal regions. The soil was a dark sandy loam. The cultivated soil was taken from a market garden close to the reserve. The samples were taken from both soils during 1928, from April to November, and from the bush soil only, during 1929, from May till November.

The process of sampling was carried out as before (2). The samples were obtained from practically the same place each time, and the soil, on being removed from the auger, was placed in sterilized tins lined with paper.

Brown's synthetic potato agar was used at pH 4·6, and the soil solution was diluted as in the previous investigation.

After determining the mean number of fungi, actinomycetes, and bacteria on the 10 plates, the probable errors of the means were determined according to the following formula:

$$\text{P.E. of mean } \frac{\sigma}{\sqrt{n-1}} \times 0{\cdot}6745$$

σ = standard deviation.

n = number of plates.

A selection of fungi from each sample was isolated on to Czapek slopes for examination later.

The mean numbers of fungi, actinomycetes, and bacteria counted for each plate are tabulated below.

TABLE 1.

Results of Samplings.

B = Bush soil; G = Garden soil.

Sample.	Date.	Soil.	Mean No. of Colonies per Plate. Fungi.	Actinomycetes.	Bacteria.
1	29.4.28	B	17·5 ± 1·53	0	5·5 ± ·42
		G	9·2 ± 2·30	0	6·7 ± ·79
2	9.6.28	B	19·3 ± 1·12	4·3 ± ·88	11·4 ± 1·39
		G	10·3 ± ·06	8·1 ± ·68	89·9 ± 1·27
3	8.7.28	B	15·2 ± ·69	12·9 ± 2·37	27·0 ± 1·39
		G	7·0 ± ·71	41·9 ± 4·24	57·6 ± 3·31
4	6.8.28	B	11·3 ± ·90	4·7 ± ·73	7·4 ± ·78
		G	9·9 ± ·97	1·6 ± ·29	17·8 ± 1·63
5	8.9.28	B	28·2 ± 1·64	8·3 ± 1·26	520·0 ± 28·37
		G	24·7 ± 4·12	27·1 ± 1·93	65·2 ± 3·79
6	14.10.28	B	12·7 ± ·75	34·9 ± 2·87	12·1 ± ·98
		G	8·8 ± ·95	39·1 ± 1·57	88·6 ± 5·14
7	25.11.28	B	13·5 ± 1·34	25·0 ± 3·0	33·7 ± 3·32
		G	9·6 ± ·70	67·8 ± 5·44	69·5 ± 3·43
8	1.5.29	B	29·0 ± 1·49	52·1 ± 1·99	17·4 ± ·81
9	15.6.29	B	18·3 ± 1·69	12·5 ± 1·02	15·1 ± 2·15
10	9.8.29	B	22·1 ± 1·81	39·6 ± 2·50	11·4 ± ·76
11	29.9.29	B	40·8 ± 1·04	90·5 ± 4·39	13·9 ± 1·78
12	2.10.29	B	35·3 ± ·84	60·4 ± 4·59	5·5 ± ·53

DISCUSSION.

The counts for the numbers of fungi and actinomycetes were plotted separately, so that the variations in numbers could be clearly seen. (See figures 1 and 2.) The numbers of bacterial colonies were very high at certain times, and could not be represented satisfactorily in a graph.

The total inches of rainfall and the mean minimum temperature readings for each month were obtained from the Meteorological Bureau. These variations were represented on paper and applied to each graph, so that the fluctuations in rainfall and temperature for each month can be seen.

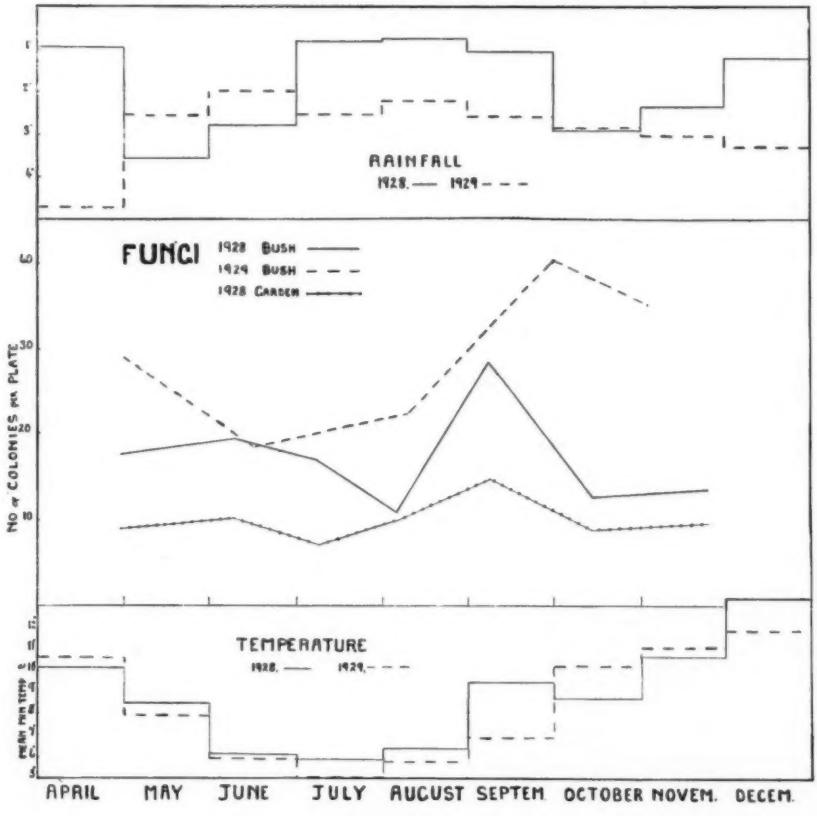

Fig. 1.

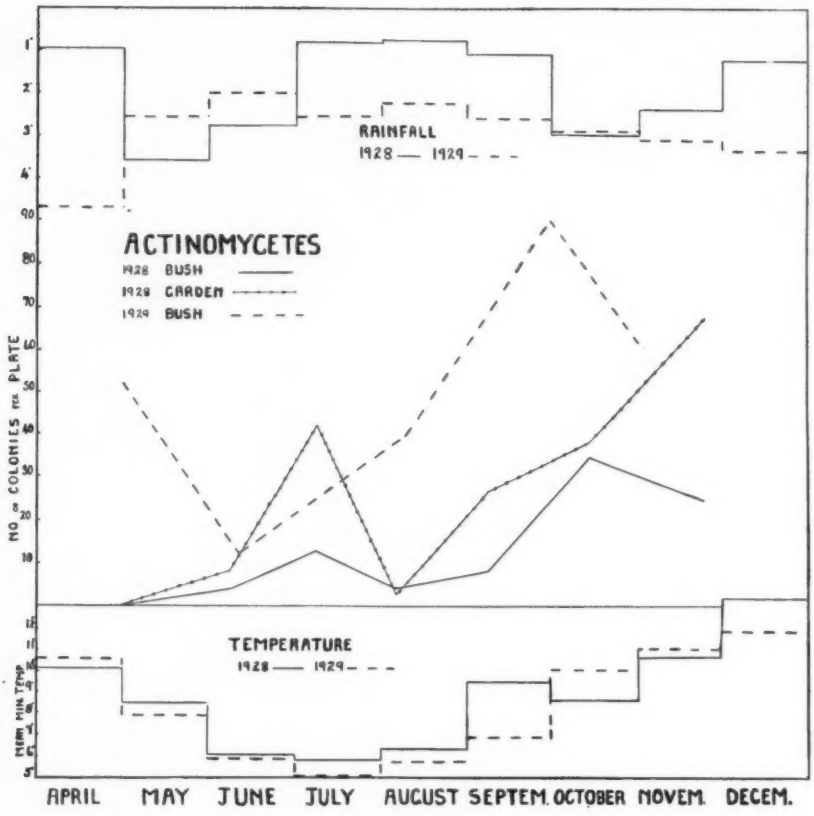

Fig. 2.

Variations in Numbers of Micro-organisms.

Fungi. Bush Soil, 1928. The fungi were more numerous on these plates than on those from the cultivated soil. A rise in numbers is seen from April to June, then a minimum in August, and a rise to the maximum count in September.

1929. The numbers during the next year were much greater on the whole, but the minimum occurred in June and the maximum late in September.

Garden Soil, 1928. It is interesting to observe that the curve for the fungi from the cultivated soil followed the one representing those in the bush soil, except that the minimum was arrived at earlier.

Actinomycetes. Bush Soil, 1928. The numbers were much lower in the bush soil than for the cultivated soil. The maximum counts occurred a month later

than for the fungi, although the minima in August coincided. There was a rise in July, but the maximum occurred in October.

1929. During this year the actinomycetes were more numerous than in the previous year, with a minimum in June and a maximum late in September.

Garden Soil, 1928. The numbers were greater and the fluctuations more pronounced, but the maxima and minima corresponded with those observed in the bush soil, except in November, when they rose considerably in the garden soil.

Bacteria. Bush Soil, 1928. The numbers of bacteria were fewer in the bush soil, except in September. During 1929 the numbers remained constantly low.

Garden Soil, 1928. The highest numbers of bacteria were found in the cultivated soil, and the maxima occurred in June and October.

1928. It can be seen that the minimum readings for the actinomycetes and bacteria correspond, that is, during late April and August. Probably the same external factors are retarding their growth.

The maxima for the different organisms in each soil do not coincide. The maxima for the fungi occurred earlier than for the actinomycetes in 1928. The bacteria followed the fungi more closely in the maximum readings, but the minima corresponded with those of the actinomycetes. The minimum in August is shown by all the organisms, except by the fungi in the garden soil, where it occurred in June.

1929. Probably a maximum had occurred in April, and a second one is observed for late September for all types of organisms. An alternation for supremacy between the types is not shown here.

The conclusion arrived at by Fleming (4), working with the fungi and bacteria in the soil, was that the relative numbers are governed by the ability of the organisms to utilize the energy present in the soil. It seems, therefore, that during 1929 there were conditions ideal for all three types at the same period, or else certain types, e.g., the bacteria, were inhibited by the environment.

Influence of Variations in Soil Moisture and Temperature during the Sampling Period.

It can be seen from the charts that there was very little rainfall in April, 1928, but that a sudden increase occurred during May and June. The months of July and August were the driest of the cold months. In September the temperature rose, but there was very little rain until October and November. The very dry period during April would account for the low numbers of all the organisms.

The rain in May and June produced definite rises in the numbers of bacteria, and later of the actinomycetes, and also slight rises in the numbers of fungi. Between July and August there was a cold, dry period, when all the

organisms were reduced in numbers, but the next fall of rain in October produced increases in the actinomycetes and bacteria in the cultivated soil.

September was a very warm month in comparison with the previous period, and the numbers of fungi appear to have been affected by the temperature. This may account for the abnormal reading for the bacteria during this month.

The numbers of fungi seem to be influenced by the temperature more than by the effect of drying and moistening, whereas the bacteria and actinomycetes increase considerably when the soil is moistened after a drying period. This was found to be the case for bacteria by Khalil (6), who worked on the effect of drying on the activity of bacteria in the soil. He says that the reduction in the bacterial content of the soil as a result of drying was followed on remoistening by a rapid increase to numbers greater than those of the soil kept permanently moist. The soil organic matter was rendered more easily decomposable by drying.

During 1929 the rainfall in April was high, and the remaining months had a good average fall, so that the soil was permanently moist. The temperature was lower from April to October, and June was the driest of the cold months.

The minima for the actinomycetes and fungi coincided, and the maximum counts were also at the same period, that is, April and late September, when the soil was warmer. The absence of fluctuations and low counts for the bacteria may be due to the absence of any period dry enough to render the soil suitable (6).

The moister soil conditions perhaps made the soil more suitable for the fungi and actinomycetes during 1929.

COMPARISON OF THE RESULTS WITH THOSE FOR THE SILURIAN AREA.

1. It has been seen that the fluctuations in numbers can be accounted for to a certain extent by the variations in soil moisture and temperature in the Tertiary sands area as well as in the Silurian clay.

2. The numbers of fungi were found to be greater in the clay soil, and there were therefore greater fluctuations than in the sandy soil. At times during 1929 the numbers in the virgin sandy soil equalled the numbers in the cultivated clay soil.

The actinomycetes were in more or less equal quantities in both areas.

3. The cultivated clay soil supported more fungi than the bush soil, while the sandy soil contained a greater number of fungi in the bush state than when cultivated.

4. The total rainfall seems to have had some effect on the total number of organisms. For example, the fungi and actinomycete counts were higher during 1927 than 1928, which was a much drier winter, and then in 1929 there was

a definite increase as compared with 1928. During 1929 all the winter months had a good average rainfall.

5. The effect of the rainfall and temperature on the fluctuations in numbers produced for both areas can be compared. The absence of rain during April in 1927 and 1928 accounts in both cases for the low counts observed for all organisms, while in 1929 the large rainfall during this month produced a larger number of micro-organisms in the bush soil, and June, which was the dry month, yielded the low counts.

The increase in rainfall at certain times affected the numbers of fungi and actinomycetes. The effect of the rising temperature in producing the increase in fungi is well demonstrated each year, especially in 1929.

A rise in temperature, combined with sufficient soil moisture, during September and October produced larger counts.

It has been stated by Khalil (6) that the bacteria increase if after a dry period they are suddenly moistened. This is seen to be probably true for actinomycetes when the graphs are studied.

As in the Silurian area, the numbers of fungi and actinomycetes were greatest on the whole about the end of the winter, that is, early spring. The differences between the exact periods can probably be accounted for by the differences in soil moisture and temperature. Erdman (3), working on Iowa soils, has found that the maximum occurred in November, 1924, that is, spring, and then decreased throughout the winter.

6. The difference between the Silurian clay soil and Tertiary sands area in supporting a larger fungal population is due probably to the differences in soil composition, rather than to the texture of the soil. Coleman (1) worked with fungi and bacteria, and found that in a clay loam the fungi were more active, while in a sandy loam the bacteria were more numerous and more active.

The difference between cultivated soil and virgin bush is thought to be due partly to the altered texture and partly to the organic and inorganic material added during manuring. The difference due to the cultivation is shown in the clay area, where the numbers and their fluctuations were larger and more marked in the cultivated soil. The sandy soil supports, when cultivated, fewer fungi than when in the virgin state, but the reverse is the case for bacteria and actinomycetes. The sandy soil was more readily dried, and therefore was incapable of supporting fungal mycelium when cultivated, but the drying rendered the soil a more suitable medium for the growth of bacteria and actinomycetes (6).

The bush soil was very acid at all times, and therefore was more suitable for fungal growth.

Higher numbers of bacteria were found by Greaves (5) in cultivated soil, and the above seems to bear out his observations, whereas Williams (8) found that with one exception the virgin soil showed higher counts.

GENERA ISOLATED.

The isolations from the plates were made more or less at random, since the medium did not give very distinct cultural characters. Any type which was noticed for the first time was selected, together with a collection representative of the 10 plates.

The genera which were determined are listed below, and the number of times species of the genus were isolated in each soil is given.

The genera which occurred in both the clay soil and the sandy soil are indicated by an asterisk.

Genera.	Cultivated Soil. 1928. 7 Samples.	Bush Soil. 1928. 7 Samples.	Bush Soil. 1929. 5 Samples.
Phycomycetes			
Mortierella*	4	3	2
Mucor*	5	3	7
Rhizopus	–	–	1
Syncephalastrum	–	–	1
Ascomycetes			
Chaetomium*	1	–	1
Fungi Imperfecti			
Aspergillus*	8	10	14
Penicillium*	8	15	13
Fusarium*	8	2	1
Spicaria*	1	–	–
Trichoderma*	1	2	3
Monilia*	–	1	–
Acrostalagmus*	1	–	–
Trichothecium	1	–	–
Corethropsis	–	–	1
Dematium	1	–	–
Hormodendrum	–	3	1
Acrothecium	–	–	1
Coniothecium	–	–	1
Macrosporium*	4	1	–
Alternaria*	1	1	–
Pestalozzia*	–	2	1
Nigrospora	2	–	–
Stemphylium	2	–	–
Phoma	1	1	1
Pleospora	1	–	–
Coniothyrium	1	–	–
	18	12	15

The difference between the types of fungi occurring in the cultivated and the bush soil can be seen. The cultivated soil appears to have supported more Phycomycetes in 1928 than the bush soil, but during 1929 *Mucors* were isolated from each sample of the bush soil. The *Aspergilli* and *Penicillia* were better represented in the uncultivated soil, and were isolated at each sampling during 1929. *Penicillia* were isolated each time in 1928. *Trichoderma*, *Hormodendrum*, and *Pestalozzia* were other genera which were well represented.

The cultivated soil showed the widest range of genera, and of these *Fusarium* and *Macrosporium* were best represented. Altogether 18 genera were isolated

during the two years, and 13 of these had been found to be present in the clay soil previously.

The presence of Phycomycetes abundantly in cultivated soil, and the predominance of species of *Penicillium*, *Aspergillus*, and *Trichoderma* in the bush soil, are in accordance with the results found in the clay soil.

Le Clerg and Smith (7) found that *Penicillia* were dominant in Colorado soil, and that species of *Aspergillus* and *Trichoderma*, as well as *Hormodendrum* and *Spicaria*, were plentiful.

SUMMARY.

1. The variations in numbers of micro-organisms are influenced by the changes in soil moisture and temperature in both the sandy and clay soils.

The minimum counts are observed during cold and dry periods of the year, and the maxima seem to occur after rain, when the temperature is high enough. During 1929 the influence of the rise in temperature can be seen best, on account of the soil being constantly moist.

2. The clay soil was observed to support a greater number of soil fungi than the sandy soil.

3. Differences brought about by cultivation were observed in each case. After cultivation the sandy soil showed a decrease in numbers of organisms, whereas in the clay soil the reverse was the case.

4. Of the genera isolated 13 were common to both areas. The uncultivated soil showed, as in the clay region, fewer Phycomycetes but a greater number of species of *Penicillium*, *Aspergillus*, and *Trichoderma*.

The investigation was carried on in the Botanical Department of the University of Melbourne, while holding the Caroline Kay Scholarship. My thanks are due to Professor Ewart for making available the facilities of the Botany Department, and also to Dr. McLennan for much helpful advice and criticism.

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THE ULTRA-VIOLET ABSORPTION SPECTRA OF THE QUINOLINE GROUP

by

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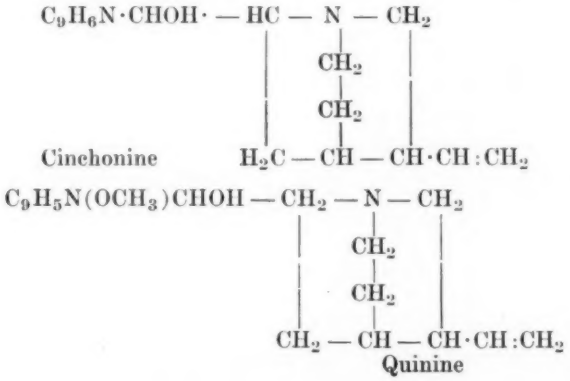
(Submitted for publication 30th August, 1930.)

The first investigations of these compounds were made by Hartley (6), Hartley and Dobbie (7), Dobbie and Lauder (2), who, by the qualitative methods then available, ascribed to cinchonine a wide band between λ3400A and λ2700A. As a preliminary to a study of the relation between structure and physiological action, this work has been extended to those substances of pharmacological interest by quantitative measurements using the Judd Lewis photometer of Hilger, and the following quantitative measurements have been made, cinchonine hydrochloride in aqueous and cinchonine in alcoholic solution being first examined. In these compounds a pair of bands is measurable in the place of the wide band of the earlier workers, while a third band appears in the region λ2200A. Comparison of the cinchonine absorption curves with those of quinoline reported by Fischer and Steiner (4), and repeated in the course of the present work, show practical identity, the quinuclidine nucleus, with its unsaturated side chain, playing no part in the molecular absorption.

This latter action is in keeping with what we know of the negligible absorption effects of saturated aliphatic rings, e.g., hexahydro-benzene. The failure of the unsaturated side chain to affect the absorption curve is to be attributed to steric hindrance.



Cinchonine

Quinine

TABLE 1.

Cinchonine in Alcohol.

	λ	$\frac{\epsilon_H}{\epsilon_V}$	log ε	
I.	3150	2·84	3·73	Maximum
	3125		2·80	Minimum
II.	2818	5·11	3·66	Maximum
	2450		2·95	Minimum
III.	2240		4·36	Maximum

Cinchonine Hydrochloride in Water.

	λ	$\frac{\epsilon_H}{\epsilon_V}$	log ε	
I.	3150	1·84	3·54	Maximum
	3125		2·80	Minimum
II.	2850	3·88	2·50	Maximum
	2470		2·95	Minimum
III.	2280		4·38	Maximum

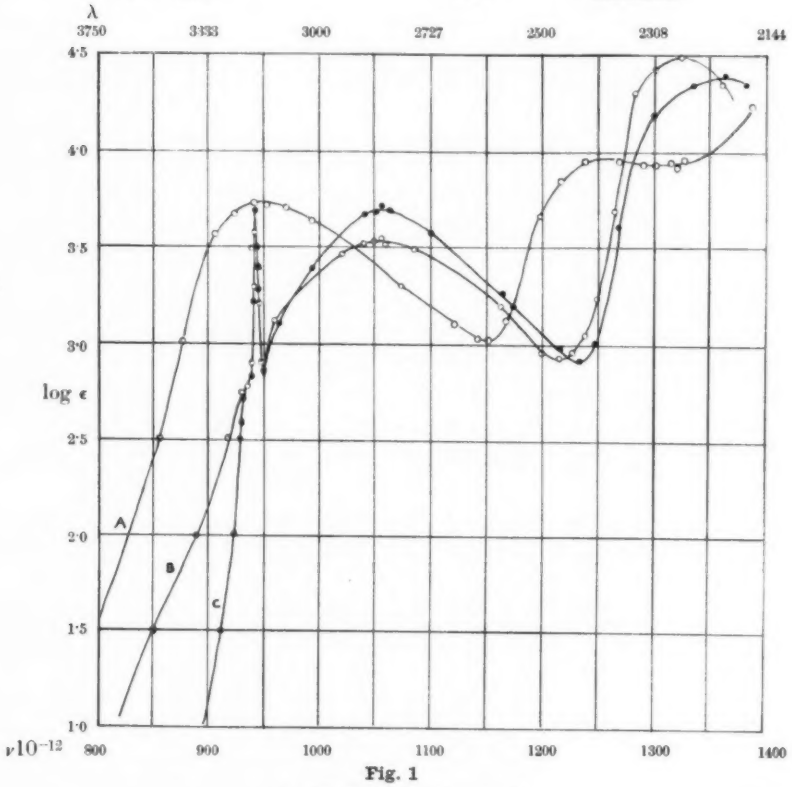


Fig. 1

A. Cinchotoxin (aqueous)
B. Cinchonin (aqueous)
C. Cinchonin (alcoholic)

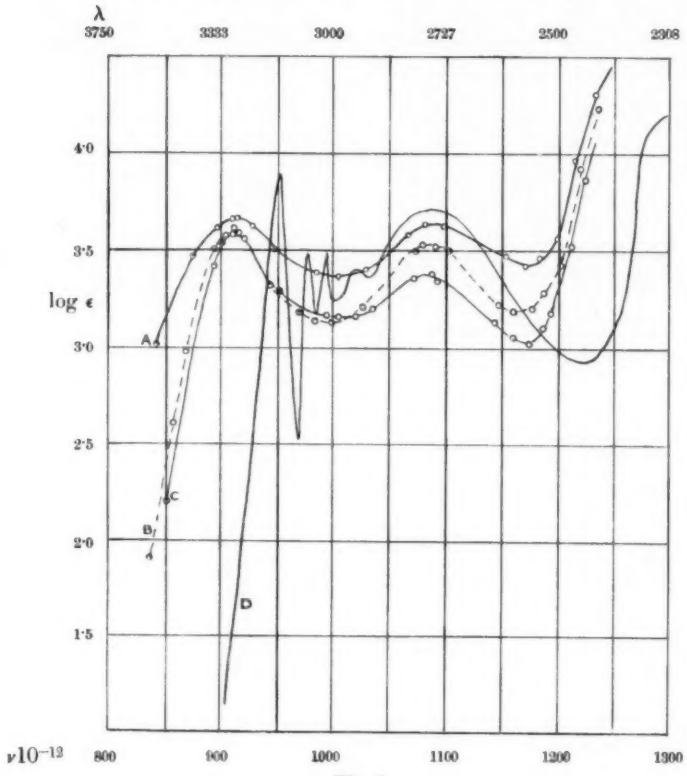
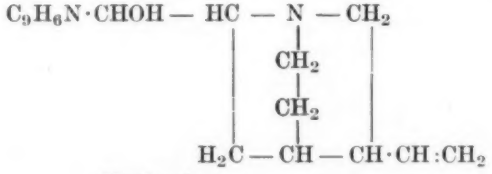


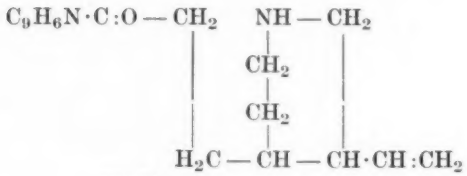
Fig. 2

A. Quinine (alcoholic)
B. Hydrocupreine (alcoholic)
C. Quinine Hydrochloride (aqueous)
D. Quinoline

The stereo-isomer cinchonidine gives practically the same absorption curve, *cf.* Dobbie and Lauder (2), while cinchotoxine, an isomer formed from cinchonine by prolonged heating with acetic acid, shows a complete change in molecular absorption, having undergone intramolecular change giving rise to a ketone and secondary base formation, involving a rupture of the quinuclidine ring.



Cinchonine



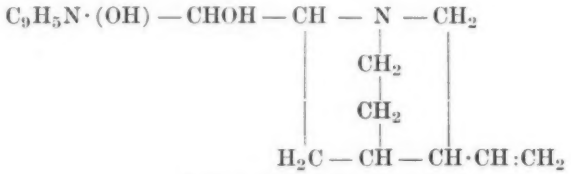
Cinchotoxin

The bands I and II of cinchonine are replaced by a wide band having a maximum about 3100A. The specific absorption is little changed, but the band is shifted some 200A towards the longer wave lengths.

λ	$\frac{\epsilon_H}{\epsilon_F}$	log ε	
3100	2·84	3·56	Maximum
2630		3·11	Minimum
2376		3·9	Maximum
2322	1·03	3·75	? Minimum
2174		4·25	

Cupreine, or Norquinine, in Alcohol.

The foundation molecule of quinine and its derivates is cupreine:



Norquinine (Cupreine)

The absorption curve has the following characteristics:

	λ	$\frac{\epsilon_H}{\epsilon_F}$	log ε	
I.	3340	1·70	3·66	Maximum
	3040		3·43	Minimum
II.	2815	1·03	3·55	Maximum
	2578		3·43	Minimum
	2190		4·95	

Compared with quinine (q.v.), it will be seen that change from —OH to —OCH_3 makes little difference to the molecular absorption, while reduction of the double band in the vinyl side chain produces, as in the case of the cinchonine molecule, no significant change, thus supporting the evidence in favour of steric hindrance in this case.

Hydrocupreine in Alcohol.

	λ	$\frac{\epsilon_{II}}{\epsilon_{p}}$	log ε	
I.	3335	2·7	3·55	Maximum
	3049		3·11	Minimum
II.	2840	2·9	3·54	Maximum
	2685		3·25	Minimum
	2413		4·73	

It makes little difference whether the CH_3 group is attached to carbon or to the nitrogen of the quinoline molecule, as evidence the following values (see figure 4) for methylquinine sulphate:

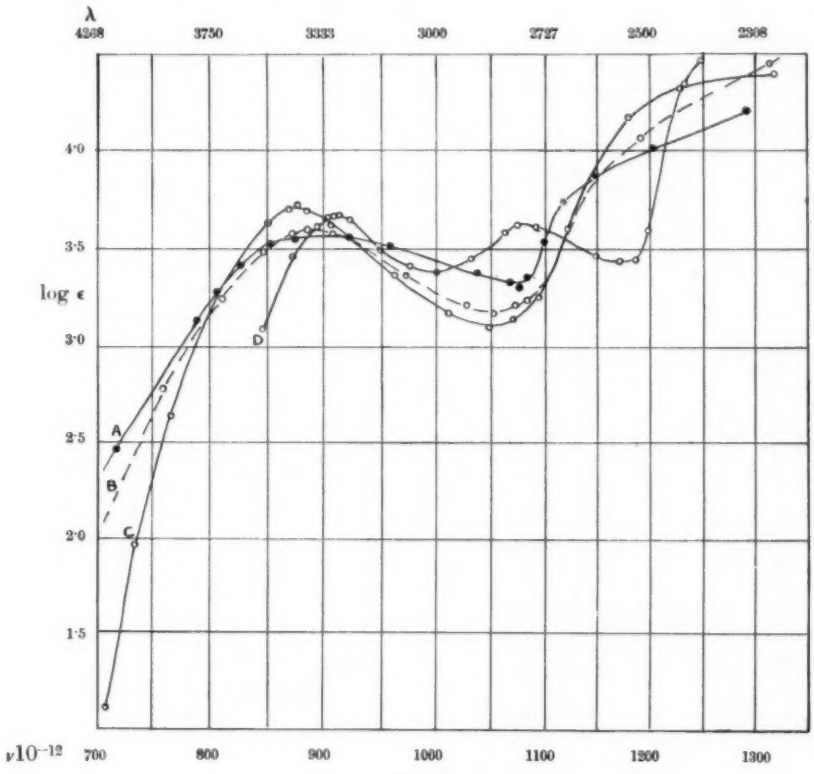


Fig. 3

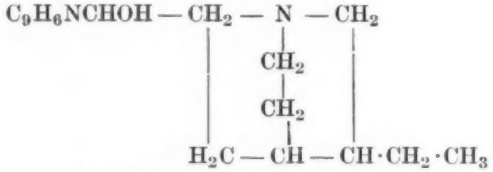
A. Chinotoxin (aqueous)
B. Hydrochinotoxin (aqueous)
C. Hydrochininone (alcoholic)
D. Quinine (alcoholic)

Methylquinine Sulphate in Water.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε
I.	3285	2·70	3·73
	3020		3·01
II.	2776	1·33	3·55
	2581		3·42
	2385		4·93

The more important hydrocuprein derivations of Morgenroth (10), introduced on account of their high bactericidal power in the presence of protein, are Optochin (ethyl hydrocuprein), Eukupin (isoamyl hydrocuprein), and Vuzin (iso-octyl hydrocuprein). It was therefore of interest to determine what change, if any, in molecular absorption is produced in the formation of these substances by substitution of H in the — OH group of the quinoline molecule.

Optochin (ethylhydrocuprein) which is quinine in which a methyl is substituted by an ethyl group and the side chain is fully reduced.



Optochin (ethylhydrocuprein)

Optochin Hydrochloride in Water.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε
I.	3355	2·48	3·82
	3400		3·43
II.	2872	1·33	3·55
	2570		3·43
	2840		4·54

The only other member of the group available was Eucupin, which gave the following values:

Eucupin Dihydrochloride in Water.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε
I.	3305	2·76	3·55
	2700		3·11
? II.	2350		3·54

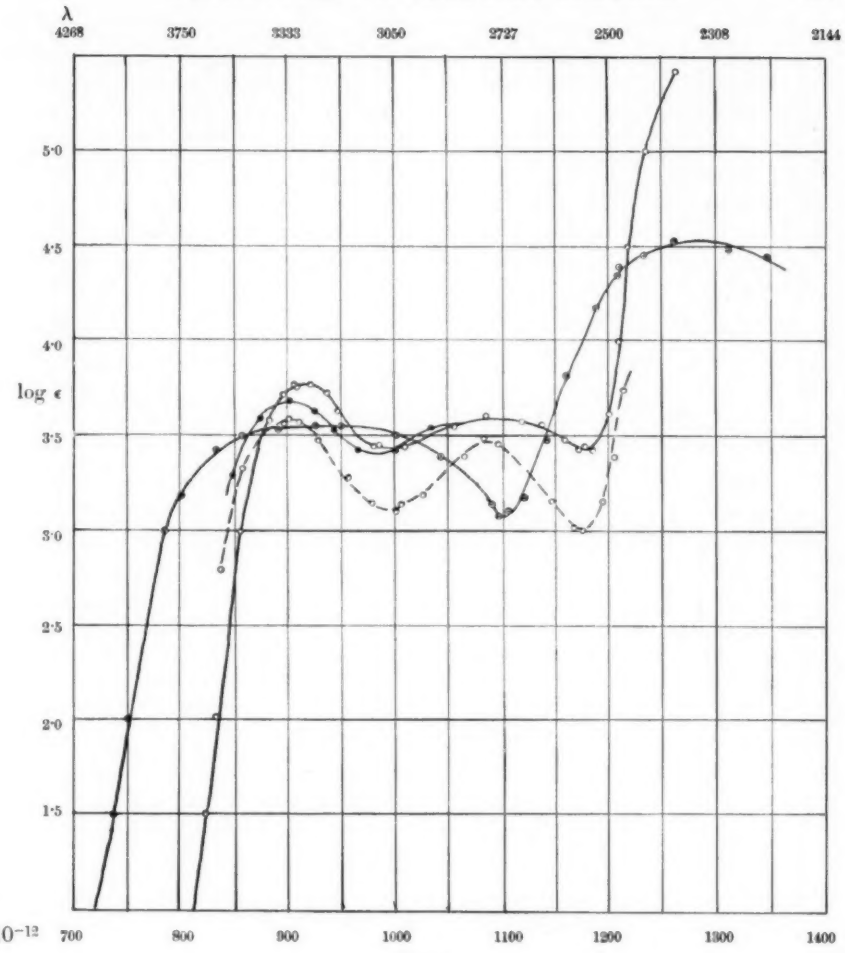


Fig. 4

Methylquinine Sulphate —— O ——
Ethylquinine Sulphate ——●——
Eucupin Hydrochloride ——Θ——
Optochin (alcoholic) — — O — — O — —

It is evident that there is a great difference in the effect on molecular stability caused by substituting ethyl by an isoamyl group. The former scarcely alters the quinine absorption curve, while the latter completely changes it, substituting for the two bands of the quinine molecule one broad band in approximately the

same position as the first band of quinine. The curve is not carried far enough into the short wave length region to determine whether there is a band at $\lambda = 2350$A, although we have determined one value at $\lambda = 2270$A, which points to the possibility of there being a maximum corresponding to that on the quinine curve in this region (see figure 4). There is a "spread" of the absorption band towards the longer wave length region.

Esterification to form quinine ethylcarbonate, or Euquinine, since it introduces no chromophore group, produces no change in absorption.

Equinine in Alcohol.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε	
I.	3305	2·61	3·67	Maximum
	3005		3·25	Minimum
II.	2800	1·75	3·54	Maximum
	2575		3·01	Minimum
	2300		4·54	

Yohimbine was measured in aqueous solution, in order to correlate, if possible, the structure of this compound with that of the foregoing. A comparison of the resulting curve with those of quinoline, and of indol Friedli (5) and Hicks (8), following the work of Barger and Field (1), shows some close relationship to both (see figure 5), but permits of no definite theoretical deduction therefrom. Winterstein and Walter (11) regard this compound as containing a condensed pyrrole-isoquinoline nucleus, and the following results are in accord with this view:

Yohimbine in Water.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε	
I.	2745	3·08	3·73	Maximum
	2445		3·24	Minimum
	2190		3·44	

Quinine.

According to previous workers (1, 2, 3) this quinine gave maxima at 3403–3035A and 2984–2606A. The sulphate, according to Dobbie (3), gave rise to a band in the region 3404–3037A. The present results are as follows:

Quinine in Alcohol.

	λ	$\frac{\epsilon_H}{\epsilon_Y}$	log ε	
I.	3268	1·70	3·66	Maximum
	3058		3·43	Minimum
II.	2805	1·30	3·54	Maximum
	2570		3·43	Minimum
	2390		4·43	

Quinine Hydrochloride in Water.

	λ	$\frac{\epsilon_{II}}{\epsilon_{\nu}}$	log ε	
I.	3295	3·39	3·73	Maximum
	3035		3·23	Minimum
II.	2800	2·06	3·54	Maximum
	2580		3·23	Minimum
III.	2325		4·25	

There may be a faint maximum in the region 2325A, but in the present work the curve appeared to run horizontally from this point (see figure 2).

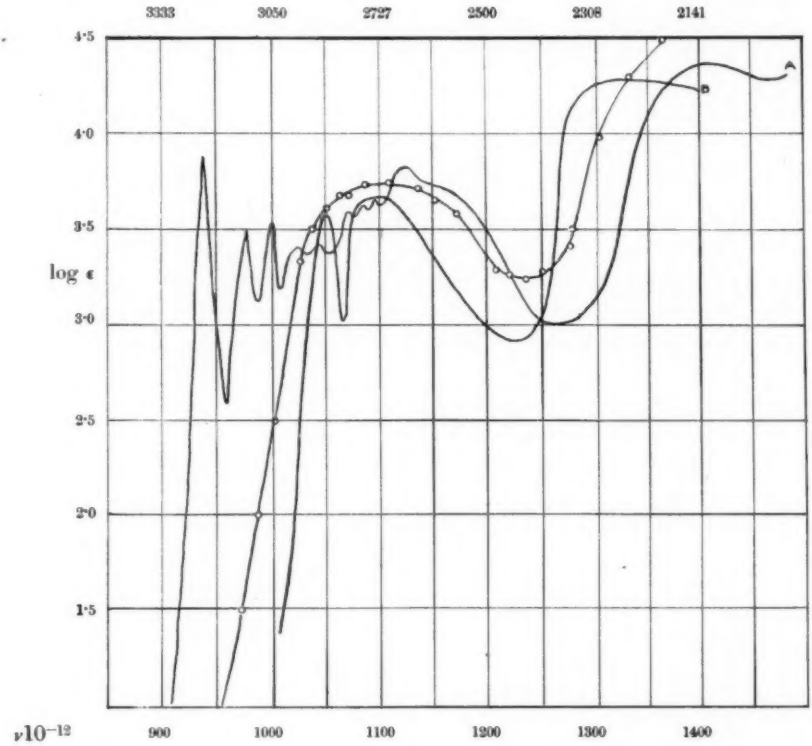


Fig. 5

Yohimbine (aqueous) — O —
A = Quinoline
B = Indol (Hexane) Friedli.

A comparison of these absorption curves with that given for quinoline by Fischer and Steiner (q.v.) shows that the small band I of Cinchonine corresponds with a similar band in quinoline. The alcoholic and aqueous solutions of quinine do not give this band. The $—OCH_3$ group in the quinine molecule, however, is responsible for a considerable shift of the absorption towards the longer wave region, and well illustrates the effect of this group when attached directly to the absorbing nucleus.

If we study the isomerism of quinine and quinotoxin, in which exactly the same intramolecular change has taken place as in the formation of Cinchotoxin, we discover the same absorption relationships. The quinine bands become wider and fuse to form a large band with a maximum about 3360A, and the absorption band spreads markedly towards the red, although the absorption coefficient remains the same. In order to trace the cause of the absorption change in cinchotoxin and quinotoxin, the cinchotoxin molecule was examined after (a) reduction of the double band in the quinuclidine side chain, and (b) oxidation of the —CHOH to —CO without opening the quinuclidine ring. The following data illustrate the significant fact that it is the —CO formation which is responsible for the change of absorption:

Hydrochinotoxin in Water.

	λ	$\frac{\epsilon_H}{\epsilon_F}$	log ε	
I.	3390	1·05	3·55	Maximum
	2865		3·24	Minimum
	2265		4·13	

Hydrochininone in Alcohol.

	λ	$\frac{\epsilon_H}{\epsilon_F}$	log ε
I.	3465	4·0	3·73
	2840		3·13
	2265		4·13

SUMMARY.

An investigation of the ultraviolet absorption curves of the cupreine derivatives of quinoline has been made as a step in the investigation of the relation between structure and physiological action.

The quinoline nucleus is the determining factor in the absorption characteristics of this group.

The quinuclidine nucleus plays so little part as to practically undisturb the absorption curve by its opening in the isomeric changes, cinchonin-cinchotoxin, and quinine-quinotoxin. Such alteration as does occur in the molecular absorption is shown to be due to the ketone formation in the connecting chain.

Reduction of the vinyl group in the side chain of the quinuclidine nucleus is without effect on absorption, owing probably to steric hindrance.

Substitution in the quinoline hydroxy group produces no change, unless it is by a long, saturated side chain, which then completely disturbs the molecular absorption characteristics.

The absorption curves of stereoisomers are practically identical.

Esterification has no effect upon the general cuprein absorption curve.

The absorption curve of yohimbine is closely related to those of indol and quinoline, which is in keeping with its dissociation into dimethylquinoline, and an ethyl or dimethylindol fragments.

In order to reduce the subject-matter, details of preparation or of working methods are not given. For the latter see Hicks and Holden (this journal (9)).

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BALANCED SALT ACTION AS MANIFESTED IN BACTERIOPHAGE PHENOMENA

by

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(Submitted for publication 18th October, 1930.)

Since the work of Ringer it has been recognized that a certain balance of inorganic ions must be maintained in the fluids used for physiological investigations if organs are to function in relatively normal fashion. Similarly Loeb and others have shown that the ova of various marine organisms require the presence in their environment of salts in concentrations resembling those found in sea-water. Other lines of investigation yielding similar results deal with the salt requirements for growth of plant seedlings and the effects of various salts on the conductivity of *Laminaria* (Osterhout). The organisms, animal or plant, which have been studied, vary in the details of their requirements, but in all cases the most obvious feature of the results is a sharply-defined antagonism between the monovalent metallic ions, represented by sodium and potassium, and the divalent or trivalent ones, particularly calcium. Thus for practically all the forms studied, pure sodium chloride solutions are definitely more toxic than distilled water, but this toxicity is completely removed by the addition of a calcium salt in amounts ranging from 1% to 5% of the concentration of the sodium salt.

Studies on the questions of ionic balance in regard to bacteria have not been very extensive or conclusive. A comprehensive review of the subject has been given by Falk (8). The clearest example of its importance is probably to be found in the greatly increased viability shown by meningococci when suspended in Ringer's solution instead of in normal saline (Flexner, 9; Shearer, 19). Except for the observation of Bordet (1), that the addition of a little extra calcium to broth favours the lytic activity of bacteriophage, there appears to be no published work bearing on the influence of salt balance on the viability or functional activity of bacteriophage or other filterable viruses.

In the course of a study of the effect of various physico-chemical agents on bacteriophages of distinct functional types, several examples of the importance of a balance between monovalent and divalent cations have been observed. These follow the general lines of the phenomenon, as it is found with higher

organisms, and may perhaps offer further support for the view that bacteriophages are essentially living independent protoplasmic units. With one exception (D44) the phages used have all been previously described from the standpoint of their biological characteristics, and were chosen to comprise representatives of the main bacteriophage types which lyse organisms of the dysentery-Salmonella group (Burnet, 3 and 4; Burnet and McKie, 6). Table 1 summarizes their main characteristics; phages grown on dysentery bacilli are prefixed by D, on bacilli of the typhoid-enteritidis-pullorum group by S.

TABLE 1.

Plaque size.	Functional type.			
	C	D	A or E	N
Large		S13	D21, D44	D13, D20, D31
Medium	D5, S18	D3	D6	
Small	S8	D4, S4	D33, S1	D26

Phage D44 has not been previously described. It is an E type dysentery phage from pig faeces, lysing smooth V, X, Y, and Z Flexner strains, inactive against W strains and all rough variants. It is the most rapidly acting dysentery phage we have examined, clearing a broth culture within two hours, and producing very large irregular plaques on agar. By the use of a method previously described (Burnet, 3), it was found that in a growing broth culture at 37° the first multiplication took place at 18–20 minutes after addition of the phage, and that each particle gave rise to 150–180 descendant particles.

1. THE INFLUENCE OF CATION BALANCE ON HEAT INACTIVATION.

The temperature at which bacteriophages are destroyed varies with different races, but in most cases lies between 70° and 75° when the phage is tested by complete immersion of a stock filtrate in a water-bath at the required temperature. If the associated constituents of the broth in which the lysed culture was obtained are removed by any of the methods for the purification of phage that have been described, or if the phage is highly diluted in normal saline, its resistance to heat is in general greatly diminished (Nanavutty, 15). Different races of phage differ in the degree to which they resist moderate heat (55° or 60°) when diluted in normal saline or in a sodium phosphate buffer, two fairly distinct groups of resistant and non-resistant phages being discernible (Burnet, 5).

Attempts were made to determine what constituents of the original filtrate were responsible for the protective action against heating, and amongst other experiments phage was diluted in buffered Ringer solution instead of the usual sodium phosphate. With the phage used (D20), exposure to 60° for 30 minutes resulted in complete inactivation of the sodium phosphate dilution, but in the

Ringer solution over 50% of the particles remained capable of producing plaques.

It was soon found that the presence of potassium in the Ringer solution was unnecessary, and that the protective effect was entirely a function of the

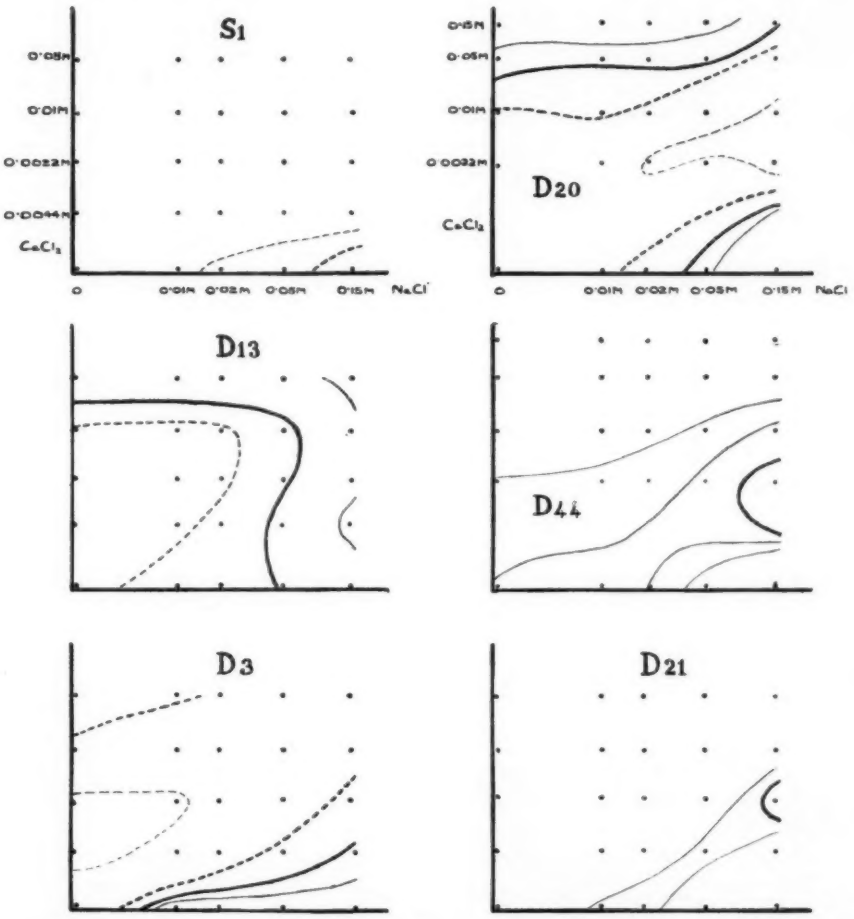

Figure 1. Percentage survival of phage particles after exposure to 60°C. for 1 hour in mixtures of NaCl and $CaCl_2$ solutions. Ordinates represent concentrations of calcium chloride, abscissae of sodium chloride. The lines indicate the proportion of phage particles surviving. In all figures the heavy continuous line corresponds to 1% survival, the heavy dotted line to 10% survival, and the thin continuous line 0·1%. The thin broken line in the case of phages D3 and D20 signifies 40% survival, but with the highly resistant phage S1 corresponds to 75% survival. Each dot represents an observation used in constructing the figures.

calcium. The phenomenon seemed to be general for all the phages which were being studied, but the degree of protection varied very considerably. Systematic experiments were therefore carried out, in which representative phages were heated for one hour at 60°C., when diluted in solutions containing varying proportions of calcium and sodium chlorides.

Experiment 1.—For each experiment a series of 25 tubes was prepared, containing in 5 cc. of glass-distilled water the following concentrations of sodium and calcium chlorides:

NaCl—0, 0·01M, 0·02M, 0·05M, 0·15M.
$CaCl_2$—0, 0·00044M, 0·0022M, 0·01M, 0·05M.

All the possible combinations of these concentrations were included in the 25 tubes, and an additional tube, containing 0·0022M $CaCl_2$ in 0·15M NaCl (Ringer proportions), served as an unheated control. In some experiments 0·15M $CaCl_2$ was substituted for the 0·00044M solution.

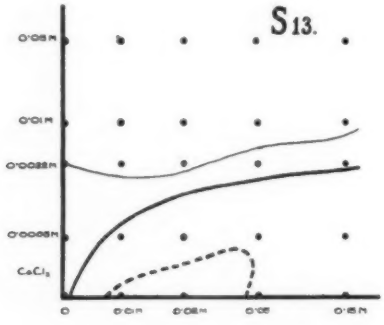

Figure 2. A similar diagram for Phage S13 exposed for 1 hour at 57°C.

Two phages, acting on different substrate organisms, were used together in most experiments. A suitable mixture of the titrated stock filtrates was made with or without the addition of sterile distilled water, the amounts of each phage being adjusted so that a convenient number of plaques should be obtained on titration of the control tube by the standard method. One drop (0·03 cc.) of the mixture was added to each tube, and the 25 experimental tubes heated for 1 hour at 60°. Each tube was then titrated by the plaque method on agar plates spread with the two substrate organisms.

The plaque counts obtained in one typical experiment are given in Table 2, and in figure 1 the results obtained with six representative phages are shown graphically.

Phage S13 was completely inactivated in all solutions at 60°. In view of

the important anomalies shown in the behaviour of this phage, a similar experiment was carried out at a lower temperature (57°), and the results are represented graphically in figure 2.

TABLE 2.

Effect of Na : Ca Balance on Survival of Phage D20 heated to 60°.

	Plaque counts.										
$CaCl_2$	0	1	0	1	0	1	0	1	0	1	
0·15M	5		2		10		5		36		
	7	0	3	0	9	0	8	0	45	0	
0·05M	41		72		116		20				
	48	0	61	0	119	0	29	0	††	9	
0·01M	††	11	††	9	††	14	††	21	†††	50	Unheated
		9		1		17		18		63	Control
											0 ††† 1 102, 91
0·0022M	††	24	†††	20	†††	44	†††	40	†††	47	
		12		27		44		34		36	
0	†††	29	†††	14	††		21		1		
		26		14		7	18	0	2	0	
NaCl	0		0·01M		0·02M		0·05M		0·15M		

0. plaques from 1 drop of original ††† confluent lysis.
1. plaques from 1 drop of original diluted $\frac{1}{170}$ †† semiconfluent plaques.

Despite the great differences in resistance to heat shown by the plaques in this series, there is a generic similarity amongst the curves. All show an obliquely directed axis of maximal survival, representing very roughly a molecular ratio Na : Ca of 50 : 1. The variations seen in the graphs spring from two sources: (a) general differences in heat resistance (*cf.* S1 with D21), and (b) differing levels of osmotic pressure being best suited for the survival of the different phages.

Most of the curves show clearly that a true "balanced salt action" is involved, both sodium and calcium being toxic to some extent when alone. For most phages, however, the calcium ion appears to be intrinsically less toxic than sodium, and with phages D3 and D13 weak calcium chloride solutions are more favourable to survival than distilled water. On the other hand calcium appears to be highly toxic for phage S13, while sodium chloride solutions, in addition to allowing partial neutralization of the $Ca^{\bullet\bullet}$ effect, afford much better survival than water alone. With this phage the protective effect of $Ca^{\bullet\bullet}$ against $Na^{\bullet}$ cannot be demonstrated. It must be remembered that in all cases small amounts of the salts from the original broth are also present, representing about 0·0005M NaCl.

The toxicity of other alkali chlorides was tested on a few phages with a similar technique. 0·15M solutions of Li, Na, K, and NH_4 chlorides were used

alone and with the addition of 0·0022M $CaCl_2$. Table 3 shows the numbers of particles remaining active in a standard drop of the mixtures after being heated for an hour at 60°. Titrations were also made of phages heated in distilled water and of unheated control dilutions.

TABLE 3.

Relative Toxicity of Monovalent Cations for Phages at 60°.

	Bacteriophages.							
	D13		D20		D44		S1	
	Alone	+Ca	Alone	+Ca	Alone	+Ca	Alone	+Ca
LiCl	6·5	9·5	1,280	9,600	6·5	4·5	7,500	9,600
NaCl	22	192	3·5	13,400	0	29	2,400	6,200
KCl	0·5	4·5	1	13,600	0	3	800	7,500
NH_4Cl			0	4,100			4,000	6,700
Aq. dest.	1,550		8,000		0		8,200	
Unheated	29,000		39,000		12,000		9,700	

Except for phage D13 there is clear evidence of an increasing toxicity with increasing atomic weight. Lithium seems to inactivate D13 a little more readily than sodium, but from the graph of this phage in figure 1 it is evident that this may only hold for the particular concentrations used. The sequence Li < Na < K is in the inverse order to that found by Loeb for toxicity toward *Fundulus* eggs, but resembles his results in so far as the effect of $Na^{\bullet}$ is the one most adequately neutralized by $Ca^{\bullet\bullet}$.

Magnesium can completely replace calcium in neutralizing the effect of the sodium ion. With phage D13 it is much more effective than calcium, but with the other three phages the protective power was approximately equivalent to that of calcium in similar concentration (Table 4).

TABLE 4.

Relative Protective Action of $Ca^{\bullet\bullet}$ and $Mg^{\bullet\bullet}$ at 60°.

Solution.	Plaque counts from phages.			
	D13	D20	D44	S1
0·15M NaCl	22	3·5	0	2,400
0·15M NaCl + ·0022M $CaCl_2$	122	13,400	29	6,200
0·15M NaCl + ·003M $MgCl_2$	3,500	12,100	35	9,300
Aq. dest.	1,550	8,000	0	8,200
Control unheated	29,000	39,000	12,000	9,700

A few experiments in which different sodium salts were studied suggested that anions had little effect apart from their possible power of reducing the effective concentration of the $Ca^{\bullet\bullet}$ ion. Phages S1 and D20 were heated to 60° in solutions containing sodium chloride, sulphate, and citrate, the concentration of $Na^{\bullet}$ in each being N/10. The same solutions were also used with the addition of $CaCl_2$ to give a concentration of 0·024%. Plaque counts are given in Table 5.

TABLE 5.

Action of Different Anions on Inactivation by Heat.

Solution.	Phage S1	Phage D20
NaCl	12,800	0
„ + $CaCl_2$	48,600	8,800
Na_2SO_4	12,200	0·5
„ + $CaCl_2$	61,200	11,500
Na_3Cit	146	0
„ + $CaCl_2$	15,600	0
Unheated control	58,700	31,700

There are no significant differences between chloride and sulphate, but there is a striking reduction in the heat resistance of S1 when citrate is present. Comparison of the results with the two phages suggests strongly that the presence of ionized $Ca^{\cdot\cdot}$ is necessary for survival in each case, but that the necessary concentration is very much greater with phage D20 than with S1. The citrate ion, however, probably has an additional action apart from its effect in reducing the amount of ionic calcium available, since equivalent concentrations of sodium oxalate are much less inhibitory when tested by this method.

Although bacteriophages survive heating in balanced salt solutions at least as well as they do when diluted in broth, the protective effect of the latter is not due entirely to its small content of ionic calcium. The addition of an excess of oxalate to broth does not destroy the whole of its protective action. The nature of the other protective substances is not certain. Small amounts of $Mg^{\cdot\cdot}$ are probably present, and in addition it has been found that reducing agents (cysteine and sodium hydrosulphite) show a slight protective effect with some phages, and that purified gelatine (0·02% in saline) has a more considerable action. It is possible that the combination of protective colloids and actual or potential reducing agents in broth may account for the residual protection apart from that due to its $Ca^{\cdot\cdot}$ and $Mg^{\cdot\cdot}$ content.

2. INACTIVATION BY DYES.

Schultz and his collaborators (Schultz and Green, 18; Clifton and Lawler, 7) have studied the action of a number of dyes on bacteriophages. Most were found to have no inactivating effect, but methylene blue and toluidine blue inactivated two staphylococcal phages. The tests were made by adding the dye to the stock broth filtrate and titrating the phage after various periods of contact at 37°. Neither dye had any action on other phages examined by this technique.

We can confirm the lack of action of methylene blue and toluidine blue on most phages when tests are made on the undiluted filtrate, but if "purified" phages were used, or the phages simply diluted sufficiently in normal saline,

many were inactivated by a 1 : 10,000 solution of either dye. When a small amount of Ca, Mg, or Ba chloride was added to the solution the inactivation was greatly decreased or abolished.

Janus green showed similar behaviour toward a few phages, but with others the inactivation seemed to be more specific, taking place indifferently in broth, normal saline, or balanced salt solutions. Since the present communication is concerned only with questions of ionic balance, the specific differences shown by functionally distinct phages in their resistance to dyes will be disregarded for the most part.

Inactivation by methylene blue.

When the phages shown in Table 1 were tested by the addition of methylene blue to the stock filtrate even a concentration of 1 : 1,000 was without appreciable inactivating effect. If plates of nutrient agar containing methylene blue were used for phage titrations the same plaque counts were obtained as on normal agar, unless the concentration of the dye was sufficient to cause visible inhibition of the bacterial growth. But when phages purified by the method of Krueger and Tamada (11) were used most of them were found to be inactivated by very low concentrations of methylene blue, and the same susceptibility was apparent when the stock filtrates were diluted in normal saline or sodium phosphate solution. Table 6 combines the results of experiments in which methylene blue (Merck, "Medicinal") was tested on purified phages suspended in normal saline and on dilute unpurified phages. The latter were diluted in phosphate solution (0·9% Na_2HPO_4 at pH 7·4), so as to give a manageable number of plaques, and, where necessary, plain broth was added, so that in all cases 1% of broth was present in the final mixture. Titrations on agar were made after 30 minutes' contact at 37°.

TABLE 6.

Plaque Counts obtained after Treatment of Phages with Methylene Blue.

Phage.	"Purified."		Dilute unpurified.		
	Control	M.B.1:10,000	Control	M.B.1:10,000	M.B.1:1,000
D3	1,200	0	37,000	18	0
D4			5,800	0	
D5			29,000	0	
D6	122	0	35,000	0	
D13	50	0	29,000	23	0·5
D20	250	0	60,000	0	
D21	68	0	4,400	0	
D26			3,100	0	
D33	250	91	45,000	200	29
S1	500	0	74,000	0	
S4	160	0	7,800	35	
S8	250	0	30,000	0	
S13	300	300	14,000	2,160	720
S18	500	0	6,000	0	

From this table it will be seen that phage S13 is highly resistant to methylene blue inactivation and D33 moderately resistant. Several other phages are not completely inactivated, but show only a very small and variable proportion of survivors. It was found that inactivation was as a rule more complete in sodium phosphate solution than in unbuffered sodium chloride. Phages D26, D4, D33, S1, and S13 regularly gave more than 0·5% of survivors with 1 : 10,000 methylene blue in normal saline, the percentage increasing in the order shown. Phages D3, D5, D6, D13, D20, D21, D31, D44, S4, S8, and S18 never gave more than 0·1% of survivors under these conditions. Results were decidedly irregular with phage S1, and part at least of the irregularity could probably be ascribed to variations in pH of the unbuffered solutions. This phage is particularly sensitive to changes in pH, being highly resistant to methylene blue in acid solution and very sensitive in alkaline. Experiments at different reactions were made in the glycine-phosphate-acetate buffer of Northrop and de Kruif (16). The results with S1 are give in Table 7.

TABLE 7.

Effect of pH on Methylene Blue Inactivation.

		Methylene blue concentration.			
Phage.	pH	1:1,000	1:10,000	1:100,000	Control.
S1	4·0	12,500	12,100	13,500	
	7·7	28	43	4,900	25,700
	9·6	0	0	6,100	

Many of the other phages showed less sharply-defined effects of pH, but when the whole series was tested for the effect of methylene blue at a pH of 4·0 a fairly clear division into groups according to the degree of protection afforded by the acid reaction could be recognized. We may summarize the results as follows:

Protected against inactivation: Phages D3, D4, D13, D26, D33, S1, S13, S18.

Slightly protected: D21.

Not protected: D5, D6, D20, D31, D44, S4, S8.

It will be found that this series corresponds almost exactly with the results of previous work on the related characteristics of ease of ascent in filter paper strips and heat resistance (Burnet, 5). Those phages which are relatively heat resistant ascend filter paper readily, and are not inactivated by methylene blue in acid solution.

Protective Effect of $Ca^{\cdot\cdot}$ and other Divalent Cations.

When phage particles are suspended in the original broth culture filtrate, or diluted in sterile nutrient broth, methylene blue has a greatly reduced or

no inactivating effect. Protection by broth, however, requires a hight concentration. A mixture containing 20% of broth in normal saline has a demonstrably slight protective action, but a 10% mixture is not protective. The active agent in broth is therefore very close to its threshold concentration. Following up the results obtained in heat inactivation experiments, it was found that small concentrations of calcium salts added to normal saline could prevent inactivation by methylene blue as effectively as nutrient broth. The protective action of calcium was shown with all the phages used in the present work except S1, with which only a minimal degree of protection was observed, and S13, the relatively high resistance of which was not increased in the presence of $Ca^{\bullet\bullet}$. Protection against methylene blue is also afforded by other divalent cations, magnesium being practically as effective as calcium.

Experiment 2. One drop of a suitably diluted mixture of phages D20 and S4 was added to each of a series of tubes containing 2 cc. of normal saline and the concentrations shown of Mg, Ca, or Ba chlorides. One drop of a 1% solution of methylene blue was added to each tube, giving a concentration of 1 : 7,500. The tubes were kept at 37° for half an hour, and their contents then titrated on the two sensitive strains. The counts obtained from a standard drop are shown in Table 8.

TABLE 8.

Protective Effect of three Divalent Cations against Methylene Blue.

0·15M NaCl + concentrations shown of Mg, Ca or Ba chlorides.	Phage S4			Phage D20		
	Mg	Ca	Ba	Mg	Ca	Ba
·01M	169	182	281	†††	†††	†††
·0032M	411	738	55	†††	†††	††
·001M	327	529	0	†††	†††	12
·00032M	0	0	0	20	31	0
·0001M	0	0	0	0	0	0
0		0			0	
Control without Methylene Blue		2,300			15,000	

In both cases calcium seems to be slightly more efficient in its protective action than magnesium and considerably more so than barium. It is interesting to note, however, that at the highest concentration tested $Ba^{\bullet\bullet}$ seems to be a better protective agent than either of the other two cations. This suggests that both the protective action over a certain range of concentrations and the inhibitory effect at higher ones are due to the one physical characteristic of the ion, both faculties being less evident with $Ba^{\bullet\bullet}$ than with $Ca^{\bullet\bullet}$ or $Mg^{\bullet\bullet}$.

Inactivation by Janus green and toluidine blue.

When dilute phages are exposed to Janus green (1 : 10,000) under the same conditions as were used with methylene blue, a smaller proportion is inactivated than is the case with the latter dye. In normal saline phages D3, D5,

D13, D20, D31, and D33 were almost completely inactivated. D6, S4, and S8 showed an intermediate degree of inactivation, while D4, D44, S1, S13, and S18 were highly resistant. Of those inactivated, D3, D20, and D31 were protected almost completely by adding 0·024% $CaCl_2$, but D13 and D33 were not protected, and D 5 only to a slight extent. With this dye it is probable that more specific factors are involved in determining whether or not inactivation will take place, but in the case of certain phages calcium has an equally well marked protective effect.

In a small series of experiments the action of toluidine blue (Merck) has been found almost identical with that of methylene blue in regard to the phages most readily inactivated, and the conditions under which inactivation takes place. Thus phages S1 and S13 are relatively resistant, D20 highly sensitive, and in an experiment similar to that shown in Table 8, in which toluidine blue 1 : 11,000 replaced methylene blue, the concentrations of the divalent ions necessary to give noteworthy protection with 0·15M NaCl were similar to those previously found, viz., Mg 0·0026M, Ca 0·001M, Ba 0·0026M.

3. THE INFLUENCE OF CALCIUM ON ELECTROPHORETIC MIGRATION RATE.

The experiments which have already been described had led to the provisional hypothesis that the effect of $Ca^{\cdot\cdot}$ is to diminish the permeability and degree of hydration of the phage particle. If such were the case it might be possible to obtain more direct evidence of a change in the size of the particle by studying the electrophoretic migration rate in solutions of constant conductivity containing varying proportions of sodium and calcium. A large number of experiments along these lines have been carried out with the simple apparatus described in another paper (Burnet and McKie, 6).

With most phages it has been found impossible to obtain sufficiently reproducible results with this technique to enable any conclusions to be drawn. By using in each experiment a mixture of phages which can be titrated independently, results lying within 20% of the mean can be obtained for the relative migration velocities of the different phages in the mixture. But when attempts were made to compare successive experiments, in which different suspending solutions were used, very much wider discrepancies appeared. These appeared to be due largely to partial inactivation of phage in the unbalanced solutions, causing an accentuation of the effect of small differences in dryness of agar, thickness of bacterial spreading, etc., on the plaque count obtained.

Phage S13, however, which has an anomalous electrical behaviour, fully described in the paper referred to, showed a characteristic and reproducible effect. In a slightly alkaline solution of sodium phosphate or chloride this phage migrates to the cathode. The amount passing into the cathodic agar is

considerably less than would be obtained if the phage were carried in passively by the electro-endosmotic flow of water. The phage particles are negatively charged, but the amount of the charge is very much smaller than is the case with any other phage we have tested.

When phage S13 is suspended in solutions of sodium and calcium chlorides of equal conductivity (0·05M NaCl, 0·026M $CaCl_2$) and pH (about 7·8), and a current of about 10 milliamps. passed for 30 minutes, it is found that in the Ca solution the direction of migration has been reversed. The results obtained in several experiments are tabulated, the figures representing the average number of plaques obtained from a standard drop of the extract made in 5 cc. of normal saline (+ a little calcium) from the agar electrodes.

TABLE 9.

	NaCl		NaCl + $CaCl_2$ (1:1)		$CaCl_2$	
	Anode.	Cathode.	Anode.	Cathode.	Anode.	Cathode.
Expt. 1.	14·5	35	30	0	21	2·5
2.	4	70	17	5	15	1
3.	8	320			25	2
4.	38	300	167	9·3		

In the presence of an excess of $Ca^{\cdot\cdot}$ the migration velocity of phage S13 toward the anode is increased sufficiently for it to overcome the counter electro-endosmotic flow, and pass in small amount into the anodic agar. The effect is not due to a diminution in the amount of endosmotic water transport. In experiment 2 of the above table the suspending media contained 0·5% glucose, and the amount of reducing substance present in the electrode extracts was determined by a modification of McLean's method. Traces only were found in the anode extracts, while the cathode extracts showed NaCl 0·0061%, NaCl + $CaCl_2$ 0·0073%, $CaCl_2$ 0·0069%.

The increased velocity associated with a replacement of $Na^{\cdot}$ by a divalent cation can hardly be due to an increased *negative* charge on the particles, and the only alternative is to presume a diminution in the effective size and electrical inertia of the particle. Whether this occurs also in the typical phages which migrate to the anode under all conditions must await a more satisfactory technique. The change in the migration velocity of S13 is relatively small, and it is only because the two rates lie on either side of a critical value (the rate of electro-endosmotic flow) that their difference can be detected by a relatively crude technique. To determine a similar increase in the case of a phage such as S1, which migrates to the anode in sodium chloride solutions about five times as fast as glucose is transported to the cathode, would require much more exactitude than has been found obtainable in the methods of phage titration.

DISCUSSION.

It is probable that every living organism requires in its environment concentrations of both monovalent and divalent cations which lie within certain limits. The requirements are stricter in the more highly differentiated organisms, but no matter what type of living cell or tissue is studied, evidence of the need for a definite balance between Na and Ca ions, or more generally between monovalent and divalent cations, is forthcoming. In the higher forms $K^{\cdot}$ and $Mg^{\cdot\cdot}$ may have specific effects which cannot be replaced by $Na^{\cdot}$ and $Ca^{\cdot\cdot}$ respectively, but there is a general resemblance amongst the alkali metal ions on the one hand and the alkaline earths on the other.

The effects of unbalanced salt solutions on living cells vary greatly with the particular organism or tissue used for the investigation, but certain general principles can be formulated. According to Lillie an excess of monovalent cations increases the permeability and reactivity of the cell, causes it to imbibe water, and often leads to inter- or intra-cellular disintegration, while on the other hand the divalent cations diminish permeability, favour the cohesive forces in the cell membrane, and diminish reactivity. In both cases the condition produced is incompatible with the normal functioning of the cell, but can be rapidly corrected by the addition of cations of the opposite type. In most cases a proportion of roughly 20 $Na^{\cdot}$: 1 $Ca^{\cdot\cdot}$ allows a normal balance between the two opposed effects.

Bacteriophages have been shown to be characteristically affected by variations in the Na : Ca ratio of the suspending medium, and when allowance is made for the enormous difference in size of the units concerned the results are essentially similar to those obtained from the study of living organisms or cells. It is naturally more difficult to investigate changes in the functional activity of a phage particle, but in so far as this is possible the results are such as one would expect if the particle were a minute protoplasmic unit, presenting toward the environment a surface essentially similar to that of a living cell. In the presence of an excess of $Na^{\cdot}$ or $K^{\cdot}$ there is an accelerated death rate, particularly at higher temperatures, and an increased susceptibility to the inactivating effect of certain dyes. Both of these actions can be partially or completely neutralized by $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, or $Ba^{\cdot\cdot}$. If we follow Osterhout in considering that one of the essential features of the death process in any cell is an increasing permeability which can be measured as a fall in electrical resistance, these phenomena fall strictly into line with the behaviour of indubitably living organisms.

In a particle as small as the bacteriophage unit we should expect changes in the permeability of its surface to be associated with relatively large corresponding changes in its size. Increase in permeability of a cell membrane is probably due to, or associated with, an increased hydration of the surface. An

increased affinity of the surface molecules of a phage particle for water must be expected to diminish its diffusibility and rate of electrophoretic migration, and conversely an apparent shrinkage in size should be associated with the decreased permeability shown in unbalanced Ca solutions.

Attempts to detect such changes by measuring the rate of electrophoretic migration under different conditions have been successful only in the case of the highly anomalous phage, S13. In solutions containing an excess of $Na^{\cdot}$ this phage migrates toward the anode at a rate too slow to overcome the opposing endosmotic flow. With excess $Ca^{\cdot\cdot}$ the rate increases sufficiently for the phage to pass into the anode, so that the change observed is a sharp apparent reversal of the direction of migration. The increase in the migration rate must represent a diminution in size or electrical inertia rather than an increased negative charge. The diminution in size is unaccompanied by any increase in the number of particles or change in their functional character, as judged by the type of plaque produced, and therefore presumably results from a simple dehydration process.

Phage S13 is in many respects anomalous, but it shows clearly the influence of Na : Ca antagonism in regard to heat inactivation, although in an unusual form (figure 2). It must remain doubtful for the present whether more typical phages also diminish in size under the influence of $Ca^{\cdot\cdot}$. The failure to detect a diminution by electrophoretic methods may be due merely to inadequacy of the titration method used. Another possibility is that phage S13, which is unusually resistant to inactivation by electropositive agents, e.g., dyes and plaster of Paris filters, may not adsorb the divalent Ca ion as readily as other phages. Any diminution in size of typical phage particles would in such event be masked by the decrease in their negative charge.

Although the evidence must remain largely indirect, we feel that it points strongly to variations in the permeability and degree of hydration of the phage particles as the cause of the characteristic behaviour toward varying Na : Ca ratios.

The various modifications of bacteriophage behaviour under the influence of different Ca : Na ratios in the environment have been shown to be of the same general type as are found with visible cells and organisms. Current views on the intimate nature of these changes in the permeability of cell membranes indicate the importance both of lipoids and of proteins (Lillie), but the relative importance of the two seems to be still undecided. All the above findings are compatible with the view that the phage particle is bounded by a surface essentially similar to other living surfaces, but in view of Loeb's studies on antagonistic salt effects on the swelling, etc., of gelatine they cannot be held to exclude conclusively the possibility suggested by Bronfenbrenner that a small inanimate phage molecule is adsorbed to particles of bacterial protein.

If our contention is justified that by alteration of the salt content of the

environment the effective size, i.e., degree of hydration and surface activity of the phage particle, can be varied to a very considerable extent, much of the discrepancy present in the literature regarding the size of the phage particle is cleared up. Bronfenbrenner and Hetler, for instance, find that phage particles in broth are smaller than when purified by electro-dialysis and suspended in buffer solution. On the other hand, Krueger and Tamada consider that phage purified by a different method is composed of smaller particles than are present in an ordinary broth filtrate. Since in the case of marine ova increase in the valence of the anion associated with $Na^{\cdot}$ accentuates the increase in permeability, a sodium phosphate buffer may be expected to increase the apparent size of phage particles very considerably. The evidence put forward by Bronfenbrenner and Hetler (2), as showing the essential independence of the true lytic agent and the particles ordinarily considered as the units of bacteriophage, can therefore be interpreted at least as readily to favour d'Herelle's view of the independent living nature of bacteriophage.

SUMMARY.

1. Bacteriophage diluted in solutions of sodium, potassium, and ammonium salts are readily inactivated by moderate heat (60°). The addition of a small amount of Ca, Mg, or Ba salt partially or completely prevents this inactivation.

2. Dilute phages, in the absence of divalent cations, are inactivated by methylene blue, toluidine blue, and Janus green. In the presence of broth or calcium salts inactivation does not occur.

3. Different phage types show characteristic individual differences in resistance to the inactivating agents and in the degree to which $Ca^{\cdot\cdot}$ prevents inactivation.

4. As judged by electrophoretic experiments the effective size of the phage particle may be considerably diminished when excess $Ca^{\cdot\cdot}$ is present.

5. The close correspondence in the effects of Na : Ca balance on the viability and functional activity of bacteriophages on the one hand, and plant or animal cells on the other, points to an essentially similar protoplasmic structure being involved in each case.

ACKNOWLEDGMENTS.

We are greatly indebted to Mr. H. F. Holden for advice on many chemical points.

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THE ELECTRICAL BEHAVIOUR OF BACTERIOPHAGES

by

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(Submitted for publication 18th October, 1930.)

It is generally accepted that bacteriophage particles under ordinary conditions carry a negative electrical charge (Todd, 18; Krueger *et al.*, 10; d'Herelle, 7; etc.), and further that the charge remains negative even when the phage particles are suspended in acid solution (pH 3·6). There are one or two contrary opinions in the literature (Koch, 8; Plantureux, 16), and it seemed possible that certain bacteriophage types might carry a positive charge, although the great majority were composed of negatively charged particles. As a large series of functionally studied bacteriophages was available, a search was made through them for phages which migrated to the cathode.

An apparatus of the type first used by Field and Teague (6), and in one or other modification by Olitsky and Long (15) and Krueger and Tamada (11), was adopted as the most convenient for a large number of experiments, although it was recognized that in such an apparatus the electro-endosmotic flow through the agar from anode to cathode would make it difficult to distinguish between uncharged and positively charged particles, which may both be carried to the cathode. But any particles carried to the anode against the endosmotic stream are indubitably negatively charged, and in being so transported are freed from uncharged or weakly charged materials accompanying them in the broth filtrate. The apparatus thus offers both a certain means of recognizing negatively charged particles and a method for the preparation of small quantities of purified bacteriophages.

Technique. Two U tubes of glass were fastened together, as shown in the diagram (figure 1), the two ends being maintained about 1 cm. apart by using rings of rubber tubing where shown. The two adjacent arms of the U tubes, along with another short glass tube, were wrapped round with cotton wool and plugged into a short, wide-mouthed specimen bottle. The two distal openings of the U tubes, and the upper opening of the delivery tube, were plugged with cotton wool, and the whole sterilized in the autoclave. When required for use the bottle was inverted in a clamp, and a sterile 0·5% solution of agar in

normal saline was run in to fill about two-thirds of each U tube. The tubes were then filled completely by adding stronger agar (1·5% in saline). In this way the central arms contained weak agar, while the outer ones contained a firmer gel. When the agar had thoroughly set the required phage solution was added with a pipette through the delivery tube. Electrical connection was made by dipping the outer ends of the U tubes into vessels containing as anode a plate of pure silver dipping into saturated NaCl solution, and as cathode a copper plate in saturated cupric chloride solution. The ordinary D.C. supply (230 V.) was used, and a suitable resistance and milliammeter incorporated in the circuit. A current of between 8 and 11 milliamps. was obtained in practically all experiments.

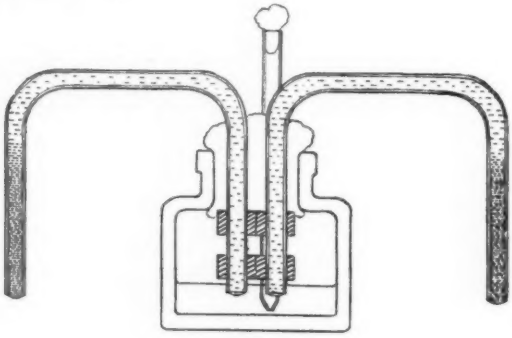

Fig. 1

After passing the current for the required period the fluid was removed and the surface of the agar washed with sterile saline through the delivery tube. The U tubes and plug were then removed, and with the aid of a pipette about 1 cm. of the soft agar was removed from each electrode into 5 cc. of sterile saline. After thoroughly breaking up the agar by drawing it backwards and forwards through a capillary pipette, the phage content of this saline emulsion was estimated by plaque counts on agar from suitable dilutions.

With such a technique the amount of phage obtained from the anode is a measure of the rate at which under the experimental conditions phage particles pass across the fluid-agar interface. This will depend on:

(a) The initial concentration of phage particles.
(b) The amount of the negative charge on each particle.
(c) The mobility of the particle, determined chiefly by its size and by the viscosity of the solution.
(d) The potential gradient between the electrodes. Since the resistance of the solution is small compared with that of the rest of the circuit, this will be proportional to the electrical resistance of the solution.

(e) The amount of endosmotic flow of water across the interface in the opposite direction.

The method is chiefly useful as a qualitative one, but if its limitations are recognized approximate quantitative measures of the rate of phage migration can be obtained with an error which is probably not larger than that inherent in the method of phage titration. The relative migration rates of two or more different phages can often be estimated by subjecting a mixture to cataphoresis and comparing the relative numbers of plaques obtained in the original mixture and from the anodic agar when titrated on the appropriate substrate organisms. In such experiments the only variables concern the phage particles themselves, their electrical characteristics, and the accuracy with which the plaque method can be applied to their titration. Experiments on the effect of the suspending medium on migration rate require the use of several sets of apparatus, and are more liable to error.

In experiments of this type titrations were made by spreading standard drops of successive tenfold dilutions in normal saline containing a little $CaCl_2$ (0·024%). From 3 to 6 drops of the dilutions likely to give a suitable number of plaques for counting (i.e., 40–400 with most phages) were plated. The degree of accuracy obtainable may be judged from the following experiment, in which a mixture of two phages was submitted to cataphoresis in three sets of apparatus under standard conditions.

Experiment 1. A mixture of phages S1 and D4, containing approximately equal numbers of each type of phage particle, was diluted (1 : 40) in 0·05M NaCl, lightly buffered by adding a drop of borate solution (pH 7·6) to each 10 cc. 10 cc. was placed in each of three electrophoresis vessels, and current passed for 250 milliamp.-minutes. The phage counts obtained from the anodic agar were as tabulated.

TABLE 1.

	Experiment i.	Experiment ii.	Experiment iii.
Phage S1.			
Dilution 2	171, 172, 177, 178, 202	158, 166, 212, 226, 254	149, 150, 158, 221, 241
„ 3	10, 15, 16, 19, 26	14, 14, 15, 16, 19	16, 17, 17, 20, 22
Phage D4.			
Dilution 2	47, 56, 63, 65, 69, 69, 72, 73	33, 43, 59, 72, 73	62, 64, 77, 81
„ 3	2, 8, 9	9, 4, 2, 2, 6	3, 7, 5, 3, 5

Titration of the original mixture submitted to electrophoresis showed a standard drop, 433,000 particles of S1 and 365,000 of D4, while the counts above correspond to an average of 18,700 and 6,140 respectively. The migration rates of S1 and D4 are in the ratio of 4·3% : 1·68%.

The plaque counts obtained from repeated platings of a standard drop of a given dilution do not give a normal frequency distribution. If the figures

obtained for the second dilution of D4 are plotted there is a peak about 70, with a sharp decline above and an irregular, more gradual fall below. An asymmetrical curve of this type is nearly always obtained when a given sample of phage is repeatedly titrated on agar. The deviation from a normal frequency curve can be ascribed to variations in the state of the substrate bacteria, depending on the thickness and regularity of their spreading, the dryness of the agar surface, the delay between the two spreadings, etc. All variations from the optimal conditions reduce the number of plaques appearing from a given number of active particles, while only gross technical error can give a plaque count in significant excess over the "real" number of particles.

In all experiments where quantitative estimations have been attempted the routine has been to eliminate any abnormally low counts and use the remainder to determine the average.

If the significance of an apparent migration to the cathode is to be estimated, it is important to have some idea of the amount of electro-endosmotic flow in the system used. This was obtained by adding a non-electrolyte (glucose) to the system, running an experiment according to the standard technique, and estimating the amount of glucose in the extracts from the agar electrodes by MacLean's method suitably modified. With 0·05M NaCl and 0·5% glucose the cathodic extract showed 0·0061%, the anode a faint trace only. Other estimations with 0·5% glucose, and other salt solutions of approximately the conductivity of 0·05M NaCl, gave 0·0069%, 0·0073%, 0·0075%. When 3·3% glucose was used, the corresponding amounts found at the cathode were 0·032% and 0·031% after the standard 250 milliamp. minutes. If the glucose is completely inert electrically, then the electro-endosmotic flow is sufficient to transport about 0·07 cc. of water across the cathode interface in the time. A completely uncharged phage would pass into the cathode agar, and a standard drop of the 5 cc. of agar extract would contain 1·4% of the number of particles present in the same volume of the original mixture. Only a value in excess of 1·4% of phage in the cathode agar will therefore allow one to conclude that it is positively charged.

THE DIRECTION OF PHAGE MIGRATION.

In view of the practically unanimous conclusion of other workers that bacteriophage particles are negatively charged at all hydrogen ion concentrations within their range of viability, it will be sufficient to state that all the following phages have shown a clear-cut migration to the anode when suspended in glycine-phosphate acetate buffer (Northrop and de Kruif, 14) at pH 7·4 and 4·2:

S1, S4, S8, S18 (*Salmonella* phages, Burnet, 1 and 2).

D3, D4, D5, D6, D13, D20, D21, D33, D44, D48 (dysentery phages, Burnet and McKie, 4).

A, B, C′, D (inactivated at pH 4·2) (*Staphylococcus albus* phages, Burnet and McKie, 3), "Wood" (*Staphylococcus aureus* phage).

Only one phage (S13) has shown any tendency to migrate to the cathode. In neutral or alkaline solutions not containing calcium salts this phage is found in higher concentration at the cathode than at the anode, but the amount present in the cathode extract has always been less than the 1·4% which represents the possible amount of passive transport by endosmosis. Some representative results are as follows:

In lightly buffered 0·15M NaCl, anode 0·5, cathode 310 (0·65%).

In lightly buffered 0·05M NaCl, anode 14·5, cathode 35 (0·054%).

In glycine-phosphate acetate buffer (pH 7·0), anode 2, cathode 60·5 (1·2%).

The electrical behaviour of this phage was strikingly affected by changes in the reaction of the suspending medium. The glycine-phosphate acetate buffer of Northrop and de Kruif was used at pH ranging from 4·2 to 8·5. A constant amount of phage was added to 10 cc. of buffer at each reaction to give approximately 4,800 particles per drop. The counts obtained from anode and cathode after 30 minutes with a current of 8–10 milliamps. are tabulated.

TABLE 2.

Behaviour of Phage S13 at different pH.

pH.	Anode.	Cathode.	Corrected Anode.
4·2	80	0·5	150
5·0	>100	1	>170
5·3	10·5	1	80
6·0	1·5	0	70
7·0	2	60·5	10
8·5	2	56·5	14

When the probable amount of electro-endosmotic flow is taken into account the migration rate to the anode may be roughly as represented in the third column. There is no reversal of sign, but in the neutral and alkaline zone the migration rate is too low to overcome the opposing endosmotic flow. Since the usual effect of increasing $H^{\cdot}$ concentration is to reduce the negative charge on colloid particles, the increased migration rate to the anode must be interpreted as due to increased mobility of the phage particle rather than increased negative charge. Shrinkage in size by elimination of attached molecules of water is the only likely method by which such increased mobility could be obtained. This interpretation is supported by the results obtained when Na is replaced by Ca in the suspending medium. As has been described previously (Burnet

and McKie, 5), a similar apparent reversal of the direction of migration of phage S13 is shown in 0·025M $CaCl_2$ solution (pH 7·6).

It is convenient to mention here some further attributes of phage S13 which also serve to differentiate it sharply from most of the other phages which we have examined:

(a) Its lytic action is strictly confined to rough strains.

(b) It is completely inactivated in 1 hour at 60°C. when diluted in balanced or unbalanced salt solutions. (When heated to 55°C. in weak calcium chloride solutions most of the phage is inactivated. This toxic effect of $Ca^{\bullet\bullet}$ can be largely neutralized by the addition of NaCl, but the usual action of $Ca^{\bullet\bullet}$ in neutralizing the toxicity of Na salts cannot be demonstrated with this phage.)

(c) It is not inactivated under ordinary conditions by any of the dyes (methylene blue, toluidine blue, and Janus green) which have been found active against other bacteriophages.

(d) Experiments on the relative diffusion rates of different phages, using a technique similar to that of Northrop and Anson (13), have shown that this phage diffuses much more rapidly than any of the others we have tested.

RELATIVE RATES OF PHAGE MIGRATION.

By using mixtures of phages which can be titrated separately it is possible to obtain a fair estimate of their relative migration rates under the same electrical conditions. In most experiments phage S1, which was found to migrate more rapidly than the other phages, was used as the standard. The plaque counts obtained from a standard drop of the anodic extract were expressed as a percentage of those present in the same volume of the original mixture. The percentage transport for each phage being determined, the relative rate of migration to the anode in terms of S1 (100) was obtained. All the experiments were carried out under the standard conditions, the diluent being 0·05M NaCl solution and current passed for 250 milliamp. minutes. The results with five phages are shown in Table 3.

TABLE 3.

Relative Migration Rates of Different Phages.

Phage.	% of S1.	Average.	Corrected Rate.
S1	100 (Standard)	100	125
D13	100, 70, 68, 61	77	102
D44	67, 53, 42, 27	47	72
D4	41, 38, 37, 33, 27	35	60
S13	—1, —13, —3·5, —8	—6·5	18·5

The electro-endosmotic flow to the cathode under these conditions is approximately 25 units, and this value has been added to give the corrected rate. The experimental error is high, particularly with the large plaque phages (D13 and D44), but the results are sufficient to show that characteristic differences in migration velocity exist.

FILTRATION THROUGH PLASTER OF PARIS.

Kramer (9) has shown that negatively charged substances are removed from solution by filtration through plaster of Paris. This offers another method of demonstrating the negative charge of bacteriophages and the anomalous character of phage S13. In these experiments it was found that small plaster of Paris filters became quickly "saturated" with phage, so that although the first few drops of a filtrate might be free from phage it appeared in the later stages. With dilute phages under standardized conditions it was found that phage S13 was the only phage to pass the filter in any appreciable amount.

Experimental. Filters were made in glass tubes of 8 mm. internal diameter by capping one end with cotton gauze and depositing on it 0·5 cc. of a thin paste containing equal weights of commercial plaster of Paris and water. The tubes were allowed to set in the vertical position, dried in the incubator overnight, suitably mounted and sterilized in the autoclave. In the filtration experiments 2 cc. of fluid was passed through each filter with a negative pressure of 100–120 mm. Hg. The phages were diluted in sodium phosphate solution (pH 7·4), so that in all cases (a) 2 drops of broth were present per 5 cc. of buffer, and (b) a countable number of plaques was given when the mixture was diluted to the standard degree (1 drop of a 1 : 160 dilution) and titrated on agar.

Under these conditions phages D4, D5, D6, D13, D20, D21, D26, D31, D33, D44, S1, S4, S8, S18 gave no plaques when the filtrate was titrated undiluted on a suitable strain. Phage D3 gave plaques representing 0·01% of the original concentration, while S13 gave 0·2%. This may be regarded as confirming the weaker negative charge of phage S13 around the neutral point, but other than electrical factors may well be involved, since even at pH 4·0 this phage passes more readily through plaster of Paris filters than any of the others tested.

ADSORPTION OF PROTEINS TO BACTERIOPHAGE.

By the use of the method of cataphoresis it is possible to study the adsorption of pure proteins to bacteriophage. Most or all phages are negatively charged at pH concentrations from 4 to 10, and may be expected to adsorb proteins carrying a positive charge, i.e., on the acid side of their isoelectric point. The two proteins used were purified gelatine and denatured globin

(kindly prepared for us by Mr. H. F. Holden) dissolved in glycine-phosphate-acetate buffer at pH 4·0. If phage were added to such a solution, and its direction of migration determined by the usual method, adsorption of protein to phage would be evident by a predominant movement of the phage to the cathode. If the usual transport to the anode occurred one could feel certain that no adsorption had taken place.

Both proteins allowed interesting differences in phage behaviour to come to light. Gelatine (purified by precipitation with alcohol at the isoelectric point after a preliminary precipitation of Ca with ammonia, washed with alcohol and ether, and dried *in vacuo*), was used in 1% solution, and the usual current (8–10 milliamps.) passed for 30 minutes. Plaque counts obtained from a standard drop of material from anode and cathode are given in Table 4.

TABLE 4.

Effect of Gelatine (pH 4·0) on Direction of Electrophoretic Migration.

Phage.	Anode.	Cathode.	Phage.	Anode.	Cathode.	Phage.	Anode.	Cathode.
D3	1,700	3,400	D20	5,130	1,190	S1	3,900	465
4	4·5	117	21	127	810	4	840	570
5	406	130	26	36	59	8	25	295
6	62	690	31	3,300	41	13	116	770
13	1,050	63	33	233	3	18	890	180
			44	24	1,300			

Practically all show a definite increase in the amount passing to the cathode, but the degree varies greatly. Phages D13, D20, D31, D33, S1, and S18 still go principally to the anode; phages D3, D5, D26, and S4 are intermediate; and D4, D6, D21, D44, S8, and S13 now migrate, or are carried by electro-endosmotic flow, predominantly to the cathode. There are thus well-marked differences in the degree to which gelatine is adsorbed by different phages.

Globin is much more reactive than gelatine at pH 4·0, and will reverse the direction of phage migration at a very low concentration. With many phages exposure to globin results in almost complete inactivation, but with those which are not inactivated the direction of migration is sharply reversed. The results with a few of the resistant phages, when tested by cataphoresis in glycine-phosphate-acetate buffer at pH4·1 with 0·2% denatured horse globin, are given in Table 5.

TABLE 5.

Reversal of Migration by Globin (0·2%).

Phage.	Anode.	Cathode.	Phage.	Anode.	Cathode.
D4	0·5	310	S13	4	2,120
D6	2	63	S18	10	478
S1	16	1,045			

The following phages are inactivated by contact with 0·2% globin for some hours, giving less than 1% of survivors, D13, D20, D31, D33; a moderate degree of inactivation, 1–20% of survivors, is shown by phages D3, D4, S4, S18; while D5, D6, D21, S1, S8, S13 are relatively unaffected with over 50% of survivors.

The specific effect of globin is demonstrable in concentrations as low as 0·02%. A comparative experiment with three representative phages is tabulated below (Table 6). A mixture of the three phages was added to solutions of globin in glycine-phosphate-acetate buffer at pH 4·0, and a current of 8–10 milliamps. passed for 30 minutes. The phage at anode and cathode, and in the original mixtures, was then titrated on the three sensitive organisms.

TABLE 6.

Action of Globin on Bacteriophages.

Concentration of globin.	S1			D33			D13		
	Control.	Anode.	Cathode.	Control.	Anode.	Cathode.	Control.	Anode.	Cathode.
0	75,000	3,400	14	30,000	173	0	15,000	535	1
0·02%	70,000	141	67	23	2	0	1,800	10·5	3
0·2%	50,000	44·5	1,510	1	0	0	750	1	19

COMMENT.

These results confirm the uniformly negative charge of bacteriophages for an extensive range of functionally distinct types of phage. It seems likely that all phages are negatively charged at pH concentrations above 4·0, although the behaviour of S13 makes it unwise to be too dogmatic on the point.

The chief point of interest in the present work is the demonstration of the existence of sharply-defined physical differences amongst bacteriophages. It has been established that phages are specifically adsorbed to the bacteria which they are capable of lysing, and not in most cases to those which are resistant to their action (d'Herelle, 7; Prausnitz and Firle, 17; Lepper, 12; Burnet, 2). These specific differences amongst phages must be related to surface configurations, which would be expected to modify also their adsorption to non-specific agents. Although it has not been possible to correlate the biologically differentiated types of phage with different forms of physical behaviour, yet amongst the types studied consistent differences of behaviour have been found in regard to:

(a) Rate of electrophoretic migration.
(b) Readiness with which oppositely charged proteins (gelatine and globin at pH 4·0) are adsorbed.
(c) Degree of adsorption by plaster of Paris filters.
(d) The effect of Na–Ca balance on inactivation by heat.

(e) The inactivating effect of dyes, particularly Janus green (Burnet and McKie, 5).

These findings are consistent with d'Herelle's view of the persisting individuality of each type of phage, and very hard to explain on any view which regards the phage particle as something simpler than a self-reproducing living unit.

The striking physical and biological differences which mark off phage S13 from all the other phages studied must be related to some deep-seated structural peculiarity. Work in progress suggests that the particles of this phage are only 1/25 to 1/100 the weight of those of most other phages, so that the negative charge must be even smaller than its slow electrophoretic rate indicates.

CONCLUSIONS.

1. Twenty representative bacteriophage types, including staphylococcal, salmonella, and dysentery phages, have all shown negative electrical charge in electrophoresis experiments.

2. One phage, lysing rough strains only, has a very much smaller negative charge than any of the others tested, and under certain conditions may pass to the cathode.

3. The electrophoretic migration rate varies characteristically for different phage types.

4. The technique provides a means of recognizing the adsorption of electrically charged agents to phage particles. Specific differences in the adsorption by different phages of two positively charged proteins (gelatine and denatured globin at pH 4·0) are described.

ACKNOWLEDGMENT.

We are deeply indebted to Mr. H. F. Holden for his advice and practical help at many points in this work.

ADDENDUM.

Since the above paper was forwarded for publication we have noted the description by Natarajan and Hyde (Amer. Journ. Hyg., 1930, vol. II, p. 652) of a large plaque coli phage showing the same anomalous behaviour in regard to pH as phage S13.

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THE IDENTITY OF THE "PRIMITIVE CELL"[1]

by

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(Submitted for publication 22nd October, 1930.)

In the mice used in this investigation the predominating form of morbid growth is a hyperplasia of the reticulo-endothelial system, or a condition closely allied thereto. As similar conditions have been reported from other laboratories, this form of metaplasia, other than being the most prevalent neoplasm found, is not a peculiarity of this strain of mice.

We have attempted in a previous communication (1) to correlate these growths, and by making a series of blood counts (2) during the life of these mice have been able (within limits) to prognosticate the particular form such neoplasmata would assume at death. Further, we have been able to indicate (also within limits) the degree of malignancy of these tumours, and fairly accurately approximate the term of life. We have found that the more primitive the cells circulating in the peripheral blood the more malignant was the condition as manifested by intenser invasion with destruction, larger growths, and earlier deaths.

These alterations in the blood picture were exhibited by increases in the number of white cells, by changes in the percentage relationships, and by the appearance of immature cells, without marked alteration in either the total numbers or the percentage average.

We have found that a polyblastic cell, which we have termed the "primitive" or "free reticular" cell, was common to all the various conditions at some stage of their development, and this is the cell we have endeavoured to identify. The difficulty lies in the fact that, although this cell has been accurately described by many authors, it still masquerades under many different names, such as "epithelioid," "histoid," "large lymphoid," and numerous others. These monoculear cells are analogous to, and probably identical with, the "resting wandering" cell of Sternberg. The same general characteristics are universally present, whether the cells are to be found in the organs, in the glands or growths, in the bone-marrow, or invading the tissues.

As far as we are able to judge, they represent the free active stage of the

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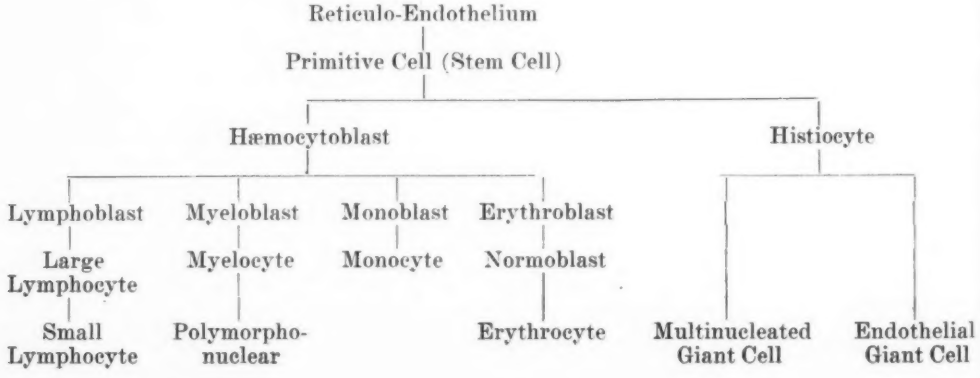
[1] This statement has been written in response to a request for more definite information upon the identity of the "primitive cell," which was described in *The Journal of Cancer Research* (1). The work was carried out in the Department of Physiology and Biochemistry, University of Adelaide, with the aid of a grant from the Commonwealth Government through the Department of Health.

fixed reticular cells, and may be seen in abnormal conditions, rounding up and freeing themselves from the reticulum in all parts of the body where the reticulum shows activity. Although from our observations a different origin does not seem possible, it is unfortunate that so much emphasis should be laid on this particular point, for Mallory (3) considers that almost every phagocytic cell is an endothelial one, while Maximow (4) contends that they are overgrown lymphocytes. Further, several investigators have suggested in recent years that, under conditions of crowding which deprive the cell of its natural share of nutriment and oxygen, and expose it to an unusual hydrogen ion concentration (product of all growth and metabolism), certain cells appear to return to more embryonal forms which are capable of life and proliferation in such adverse circumstances.

Our observations, although at present limited to stained sections of fixed tissues (where although certain alterations may take place in given cells, such alterations are the same for the same type of cell wherever found), would strongly suggest an overstimulation of the reticulum, due to some at present unknown factors. This stimulation causes an overproduction of such intensity that crowding results, and these comparatively "primitive" cells, losing their power to differentiate, are thrust into the blood stream and tissues at such a rate that they soon displace all other cells, *vide* the leukemias and lymphosarcomata.

There is season to believe that the hydrogen ion concentration, due to crowding, may exercise some effect on the loss of a cell's capacity to differentiate, for in the less dense portions of these growths and invasions of lymphoid metaplasia a certain proportion of differentiated cells are invariably present. These free reticular cells, retaining the polyvalent character of embryonic cells, possess the latent potentiality of differentiating into polyblastic cells, viz., the hæmocytoblastic parent cell of the white blood corpuscle, or of hypertrophying into the mononuclear phagocytes, viz., the parent cells of the endothelia and multinucleated giant cells of the bone-marrow and tissues, thus:



The levels between the differentiating cells are not abrupt, and the change evolves gradually, so that, at certain stages of transition, one could not definitely state: "This is a lymphoblast," or "This is a free reticular cell." The transitions are so gradual, through so many intermediate forms, that hard and fast definitions are difficult. With the advancement of differentiation, the classification becomes less complex. This is clearly seen in the bone-marrow when cells are developing at a greater rate than is normal.

Most investigators concede that the nucleus of this cell is larger than that of the fixed reticular cell; that in fixed and stained sections this nucleus is vesicular, pale, and polychromate, with conspicuous interlacing chromatin threads and a prominent nucleolus; that the cytoplasm, especially where the cells are massed together, appears scanty and faintly basophilic in reaction. Granulations are absent and the peroxidase reaction is negative. Few positive characters distinguish this cell, although it is of definite type and fairly uniform in size and shape.

CONCLUSIONS.

1. It appears that the free reticular cell is the true hæmocytoblast of the monophyletists, and by differentiation becomes the polyblastic cell, which is the common ancestor of all the blood corpuscles; this cell then must be the most primitive cell capable of production by adult hæmatopoietic tissues.

2. This cell shows polyvalent potentialities, and according to environment (possibly of a chemotactic nature), differentiates into either a polyblastic stem cell with capacity for further differentiation, or into the large phagocytic giant cell, which may eventually become multinucleated; therefore this direct derivative of the reticulo-endothelium has retained the primordial polyvalent character of the mesochymal cells of embryonic tissue.

3. If "as a general rule cells of malignant tumours lose their physiological function, and devote themselves entirely to growth," these cells may acquire this characteristic of malignancy.

4. As an embryonal type of cell is indicative of, or at least one of the criteria for, a definition of malignancy, then when this free reticular cell becomes the predominant type a certain degree of malignancy must be indicated.

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THE INHERITANCE AND LINKAGE RELATIONS OF XANTHA SEEDLINGS IN MAIZE

by

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(Submitted for publication 29th October, 1930.)

REVIEW OF LITERATURE.

The chlorophyll deficiency character of maize seedlings known as "xantha" was first investigated by Trajkovitch in 1924 (3). It was shown that the yellow xantha seedlings contained about 10·7% of the normal amount of chlorophyll and contained the normal amount of xanthophyll. By means of a cross involving two homozygous virescent plants and two plants heterozygous for the xantha character, it was shown that the factor or factors for xantha seedling character are different from the factors controlling virescent or yellow (luteus) seedling. As the progeny from selfed plants, having the same selfed ear as a parent, showed both 3 : 1 and 15 : 1 ratios, it was strongly indicated that the xantha seedling character was determined by duplicate genes. This fact was not confirmed by further investigation, however. Further, Miss Trajkovitch demonstrated that the gene for xantha seedlings was inherited independently of the gene for virescent seedlings and the gene for yellow endosperm.

MATERIALS AND METHODS.

Pedigree No. F2084 (Table 1) was the result of a cross between black pop corn and Miss Trajkovitch's xantha seedling (Tr. 188-7), made by Dr. H. C. Fraser, of Cornell University, in 1925. Coloured and colourless endosperm seeds from the three ears of pedigree 2084 were grown at Cornell University by Dr. I. F. Phipps in 1927. The resulting plants were selfed and, in addition, three back-crosses for endosperm colour were made. Several crosses were also made with R-testers and C-testers.

To investigate linkage, those plants which showed evidence of linkage in 1927 were grown at the Waite Agricultural Research Institute in 1928, and a further number of back-crosses was made for endosperm colour.

GENETICAL ANALYSIS.

The seedling counts of the three ears from pedigree 2084 are shown in Table 1. Pedigrees 2084-1 and 2084-3 were segregating green to xantha seed-

lings in a 15 : 1 ratio, and pedigree 2084-2 in a 3 : 1 ratio. These results tend to confirm the presence of two duplicate genes determining the xantha seedling character. These genes have been designated Xn_1xn_1 and Xn_2xn_2 by Trajkovitch (3).

The seedling counts and data on aleurone and seedling colour for progeny of the selfed ears of pedigree 2084, together with the three back-crosses, are grouped in Table 2. The segregation for xantha seedlings occurred in either the 15 : 1 or the 3 : 1 ratios. All progeny from pedigree 2084-2 segregated in a 3 : 1 ratio.

The back-crosses segregated for coloured and colourless endosperm in the ratios 1 : 1 or 3 : 1, and the selfed plants segregated for endosperm colour in either a 9 : 7 or 3 : 1 ratio. Of the three factors for endosperm colour, A, C, and R, A was present in the parent material in the homozygous dominant condition, and was not segregating in any of the progenies grown. The 9 : 7 ratio indicates the segregation of two factors for endosperm colour, and these factors then must be C and R. Where the 3 : 1 ratio indicates the segregation of one factor for endosperm colour, this factor may be either C or R. However, in all those plants which showed the 3 : 1 ratio, except pedigree 4088A-2, seeds with mottled endosperm appeared among the coloured seeds. As mottling is expressed only in the presence of heterozygous R, and only when R is contributed by the male parent, the evidence is conclusive that the one factor for endosperm colour segregating in those plants showing the 3 : 1 ratio is R, and not C. In the ear 4088A-2 the purple endosperm colour was very poorly developed and the coloured seeds could not be classified into purple and mottled.

The genetic constitutions of pedigree 2084 may be expressed as follows:

Pedigree 2084-1, „ 2084-3 } AACcRr Xn_1xn_1 Xn_2xn_2

„ 2084-2 AACcRr Xn_1xn_1 xn_2xn_2

Of the progeny resulting from the selfed ears of pedigree 2084, those showing linkage with R, and giving the 3 : 1 ratio for endosperm colour and 3 : 1 for xantha, would have the constitution AACCRr Xn_1xn_1 xn_2xn_2; those giving 9 : 7 and 3 : 1, AACcRr Xn_1xn_1 xn_2xn_2; those giving 3 : 1 and 15 : 1, AACCRr Xn_1xn_1 Xn_2xn_2; and those giving 9 : 7 and 15 : 1, AACcRr Xn_1xn_1 Xn_2xn_2.

LINKAGE TESTS.

Linkage of R with Xn.

Those plants grown in 1927 which showed any evidence of linkage between a factor for xantha seedling and the aleurone colour factor R were continued to the next generation, and a further number of back-crosses and intercrosses was made in 1928. Coloured and colourless seeds from the selfed plants

and crosses were planted in 1930, and seedling counts made. The results are summarized in Tables 3 to 8, and indicate a definite linkage between one of the xantha seedling factors and the endosperm colour factor R.

In Table 3 are shown the linkage data for six selfed plants segregating in the ratios 3 : 1 for endosperm colour and 3 : 1 for xantha. The endosperm colour factor segregating is R, as shown above, and an average of the cross-over percentages gives 31·5% of crossing over, the X^2 value being 3·1101. The cross-over percentages were all calculated by means of Fisher's produce formula (1):

$$\frac{ad}{bc} = \frac{2p^2 + p^4}{1 - 2p^2 + p^4}$$

where p = cross-over percentage in repulsion series
and $1-p$ = cross-over percentage in coupling series
ad = the product of the phenotypic classes AB and ab
bc = the product of the phenotypic classes Ab and aB.

No linkage in the repulsion series was found for the 3 : 1, 3 : 1 ratios.

In Table 4 results from two back-crosses showing linkage in the coupling series are shown. As the results came from back-crosses the endosperm colour ratio is 1 : 1. The back-cross results show a lower cross-over percentage than the selfed plants of Table 3, the average cross-over percentage being 22·5%, which, when compared with the 22% calculated values, gives $X^2 = 1·0436$. The results of a back-cross showing linkage in the repulsion series and 1 : 1, 3 : 1 ratios is shown in Table 5, and the cross-over of 21·6% agrees fairly closely with the average cross-over percentage from Table 4. The cross-over percentages for the 1 : 1, 3 : 1 ratios were calculated by means of Immer's product formula (2) $\frac{ad}{bc} = \frac{p + p^2}{2 - 3p + p^2}$, where the symbols have the same meaning as described above.

Results showing linkage in the coupling series for plants segregating in ratios 9 : 7, 3 : 1, are shown in Table 6. The data come from three selfed plants and two back-crosses, and give an average cross-over percentage of 30·5%, which is close to the average for the selfed plants of Table 3. The linkage results in the repulsion series for 9 : 7, 3 : 1 ratios in Table 7 are, for some unknown reason, remarkably low, giving an average of only 10·85% crossing over. The cross-over percentages for the 9 : 7, 3 : 1 ratios were calculated by means of Immer's product formula (2): $\frac{ad}{bc} = \frac{2 + 7p^2 + 3p^4}{6 - 9p^2 + 3p^4}$

Those plants which gave no indication of linkage between R and Xn are grouped in Table 8. Pedigree 4093-7 x 41062 showed mottled endosperm seeds. The R factor is therefore segregating, and as no linkage was found it is concluded that the second xantha factor (xn_2) is segregating. Pedigree 4087B-2 x A-8 does not show mottled endosperm seeds, and, as R is thus not segregating, the factor C must be segregating. In the remaining pedigrees in the table R is

segregating, and the observed independent inheritance is assumed to be due to the segregation of xn_2 instead of xn_1.

An average of all linkage results, including the very low result of 11% for the repulsion series of 9 : 7, 3 : 1 ratios, gives a cross-over percentage of 23·4%. Omitting the abnormal 11% raises the percentage to 26·4%.

SUMMARY.

Coloured and colourless endosperm seeds from a hybrid which was segregating xantha seedlings were grown and carried through several generations. The presence of two duplicate genes, xn_1 and xn_2, determining the xantha seedling character was confirmed.

Plants showing evidence of linkage between the endosperm colour factor R and one of the factors xn_1, for xantha seedling, were carried on through several generations and a number of back-crosses for endosperm colour made. A definite linkage between R and xn_1 was found, an average of results giving a cross-over value of 23·4%. The linkage was found in both coupling and repulsion series, and for 1 : 1 and 3 : 1, 3 : 1 and 3 : 1, and 9 : 7 and 3 : 1 ratios. Omitting a very low result of 11% for the repulsion series of 9 : 7 and 3 : 1 ratios, the cross-over percentage is raised to 26·4, which seems a better average of the results than 23·4%.

ACKNOWLEDGMENTS.

The writer wishes to express his grateful appreciation to Dr. I. F. Phipps, who made the preliminary crosses at Cornell University, and later crosses at the Waite Agricultural Research Institute, and through whose kindness and help the writer was able to continue the work and complete the problem.

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TABLE 1.

Parent plants heterozygous for xantha.

Pedigree Numbers.	Green.	Xantha.	Total.	Ratio.	Deviation ÷ P.E.
2084–1	95	4	99	15:1	1·3
–2	96	17	113	3:1	3·6
–3	89	5	94	15:1	·55

TABLE 2.

Progeny of selfed ears of Pedigree 2084, and three back-crosses for endosperm colour.

Pedigree No.		Aleurone colour and seedling colour				Ratio			
		Coloured.		Colourless.		Coloured :	Devi-		Devi-
1925.	1927.	Xn	xn	Xn	xn	Colour-less.	ation ÷ P.E.	Ratio Xn : xn	ation ÷ P.E.
2084–3	2544A–5	31	10	—	—	—	—	3:1	·13
	2544C–1 × 2546A–5	60	17	23	5	3:1	·58	3:1	1·42
	2544C–10 × A–4	44	6	44	21	1:1	2·07	3:1	1·04
	2544B–1 × A–5	43	12	—	—	—	—	3:1	·80
	2544A–6	—	—	30	—	—	—	—	—
	B–5	—	—	78	—	—	—	—	—
	C–4	108	—	40	—	3:1	·84	—	—
	C–8	60	—	13	—	3:1	2·1	—	—
	C–11	69	—	57	—	9:7	·48	—	—
	C–14	69	—	19	—	3:1	1·09	—	—
2084–2	2545A–1	11	2	12	6	9:7	2·14	3:1	·15
	A–2	41	6	11	4	3:1	·21	3:1	2·74
	A–13	42	7	15	10	9:7	2·56	3:1	·59
	A–14	42	9	36	11	9:7	1·24	3:1	1·21
	A–5	43	—	21	—	3:1	2·13	—	—
	A–9	29	—	—	—	—	—	—	—
	C–6	—	—	25	9	—	—	3:1	·29
	C–7	—	—	48	6	—	—	3:1	3·49
2084–1	2546A–4	24	1	14	10	9:7	1·04	3:1	·61
	A–16	47	9	33	—	9:7	1·78	15:1	2·23
	A–18	49	7	21	3	3:1	1·53	15:1	3·4
	A–21	78	5	28	7	3:1	1·74	15:1	2·61
	A–1	62	—	17	—	3:1	1·06	—	—
	A–2	19	—	9	—	3:1	1·28	—	—
	A–14	66	—	25	—	3:1	·80	—	—
	A–15	60	—	12	—	3:1	2·41	—	—
	A–20	50	—	12	—	3:1	1·52	—	—
	B–3	—	—	51	14	—	—	3:1	·95
	B–7	52	6	30	9	9:7	1·04	3:1	3·21
	B–11	63	5	10	9	3:1	1·01	3:1	2·84
	B–12	—	—	78	30	—	—	3:1	·98
	B–10	72	—	29	—	3:1	1·27	—	—

TABLE 3.

Linkage in the coupling series; selfed plants segregating in F_2 in the ratios 3 : 1 for colour of endosperm and 3 : 1 for xantha seedling.

Pedigree No.							
1927.	1928.	RXn	Rxn	rXn	rxn	Total	% Cross-overs.
2545A–2	4094A–7	59	10	14	11	94	30
2546B–11	4088A–4	35	15	7	7	64	38
	4088A–2	47	7	5	10	69	20
2546A–21	4087A–7	49	9	8	6	72	31·5
	4087A–1	35	13	9	8	65	38
2545A–13	4090B–13	37	11	5	6	59	31·5
Totals and average % . . .		262	65	48	48	423	31·5
Calculated on basis of 32%		260·39	56·85	56·85	48·89		

$X^2 = 3·1101$

TABLE 4.

Linkage in the coupling series; back-crosses segregating in the ratios 1 : 1 for endosperm colour and 3 : 1 for xantha seedling.

Pedigree No. 1927.	1928.	RXn	Rxn	rXn	rxn	Total	% Cross-overs.
4088B–2 × 4088A		38	4	18	12	72	18
4089–3 × 4088B–1		38	4	31	12	85	27
Totals and average % . . .		76	8	49	24	157	22·5
Calculated on basis of 22%		69·86	8·63	47·88	30·61		

$X^2 = 1{\cdot}0436$

TABLE 5.

Linkage in the repulsion series; back-cross segregating in the ratios 1 : 1 for endosperm colour and 3 : 1 for xantha seedling.

Pedigree No.	RXn	Rxn	rXn	rxn	Total	% Cross-overs.
4087A–3 × B–1	55	30	68	7	160	21·6
Calculated on basis of 22% . .	48·8	31·2	71·2	8·8		

$X^2 = 1{\cdot}3458$

TABLE 6.

Linkage in the coupling series; plants segregating in ratios 9 : 7 for endosperm colour and 3 : 1 for xantha seedling.

Pedigree No. 1927.	1928.	RXn	Rxn	rXn	rxn	Total.	% Cross-overs.
2546B–4 × A–15	4092A–1	38	10	17	13	78	28·5
2545A–13	4090B–1	47	13	21	16	97	30
	4089A–1 × 4088B–1	42	11	30	15	98	36
	4089–2 × 4087B–6	20	4	12	6	42	31
2544B–1 × A–5	4093–4	65	10	31	16	122	27
Totals and Average %		212	48	111	66	437	30·5
Calculated on basis of 30%		204·02	41·78	123·72	67·46		

$X^2 = 2{\cdot}5771$

TABLE 7.

Linkage in the repulsion series; plants segregating in the ratios 9 : 7 for endosperm colour and 3 : 1 for xantha seedling.

Pedigree No. 1927.	1928.	RXn	Rxn	rXn	rxn	Total.	% Cross-over.
2305–6 × 2545A–1	4096–4	37	24	45	10	116	7
2544B–1 × A–5	4093–5	46	21	44	6	117	14·7
Totals and Average %		83	45	89	16	233	10·85
Calculated on basis of 11%		87·9	43·15	86·84	15·09		

$X^2 = \cdot 4822$

TABLE 8.

Plants showing independence inheritance.

Ratio.	Pedigree No. 1927.	Pedigree No. 1928.	RXn	Rxn	rXn	rxn	Total	X^2
1:1, 3:1		4093–7 × 4106–2	40	11	42	12	105	·3765
		4087B–2 × A–8	22	7	17	12	58	4·1598
3:1, 3:1	2545A–2	4094–2	29	12	12	8	61	3·6244
	2546A–16	4091–2	38	13	16	4	71	·6815
9:7, 3:1	2545A–13	4090B–8	12	3	10	3	28	·3809
	,,	4090B–12	25	11	13	9	58	4·0371
	2546B–4	4092A–8	19	6	12	5	42	·3426

Note: X^2 value of 9·0000 for four classes gives odds of 34:1.

THE TOXICITY OF BLOOD WHICH HAS BEEN FROZEN

by

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(Submitted for publication 11th November, 1930.)

That blood undergoes haemolysis when frozen and subsequently thawed is a commonplace of the text-books. As usually stated repeated freezings and thawings are necessary. Other less obvious changes are also produced. The late Professor N. Alcock discovered in 1903 that blood which had been frozen and thawed had a lowered electric conductivity. This result he communicated to me verbally, but he did not pursue the matter, and I doubt if any announcement on the subject was published under his name. In collaboration with Miss Lilias Jackson (Mrs. Maxwell) I carried out a number of experiments on this curious change of conductivity, but abandoned the investigation, partly because Professor G. N. Stewart was engaged on the same problem.

The only irreversible changes conceivable which could be produced by the freezing and thawing of a homogeneous true solution would be those dependent on the fact that the solution was supersaturated with respect to some constituent. This substance might be thrown, in part, out of solution, and the excess above saturation would not be redissolved. It is also possible that certain colloids could be precipitated either by their own concentration or by the concentration of electrolytes during the freezing process. It is said that some wines are altered in flavour by being frozen—which of these alternatives might here be operative is an interesting question. But when we are dealing with formed elements, when we are confronted with the problem of structure, we can recognize at once that changes of a far-reaching character may be produced by gelation. The irreversible alteration in blood which we designate haemolysis has attracted considerable attention, but I have been concerned in this investigation with a property of the frozen-thawed blood which has not, to my knowledge, been described before, and that is the toxicity of such blood when returned to the donor by intravenous injection.

METHODS.

Dogs were given a preliminary hypodermic injection of 4 grains of morphine, and were then subjected to full chloroform anaesthesia. Blood was taken from a cannulized carotid, and was immediately defibrinated. It was then filtered through several ply of muslin to remove any fibrin threads. About 25 cc. were kept as a control, and the rest frozen, thawed, refrozen, and rethawed. The carotid pressure of the dog (still under anaesthesia, of course) was taken from the same artery which had yielded the blood previously, and the external jugular vein on one side was cannulized for injection purposes. All blood injected was at body temperature. In some cases the vagi were cut, in others they were left intact; the same results were always obtained.

RESULTS.

A control of 20 cc. defibrinated unfrozen blood was injected. The carotid pressure showed no change or a trifling rise. When, however, 20 cc. of the animal's own blood, which had been doubly frozen and thawed, were injected there was a syncopal fall of blood pressure, with exaggerated breathing. Sometimes death resulted; but when recovery occurred it took a fair time to be established, and was never complete in the time allotted to an experiment. Plethysmographic records showed that there was arteriolar dilatation, but the heart was directly affected, and such was the cause of death when this took place.

Some curious differences were observed, according to the manner in which the blood had been frozen. If the blood were contained in a single large test-tube of 1¼ inch internal diameter the toxicity was much greater than if the blood had been frozen in several test-tubes of the usual laboratory size.[1] I made no quantitative determinations of haemolysis, but in those cases where there was feeble toxicity the eye alone could easily perceive that the haemolysis was slight. Of the many records of experiments obtained I choose two (figures 1 and 2), where the syncope was not lethal. These records are reduced in size, as evidenced by the centimetre scale at the side of each figure.

My first idea was that the phenomenon was due to embolism caused by the ragged "ghosts" of the broken corpuscles. To investigate this the frozen and thawed blood was centrifuged a couple of hours, until a clear though red serum was produced. A simple test of the efficiency of the centrifuging was to place a few cc. of the serum in two cylinders, one containing distilled water, the other 0·9% NaCl. If any suspended matter was present a difference in the optical appearance of the two cylinders, placed in sunlight and gently agitated, was

[1] This may explain the anomaly that when I froze the hind leg of the fully anaesthetized dog by immersion in ice-salt mixture I obtained no evidence of toxic action when the limb was thawed. During the freezing both femoral artery and vein were ligatured.

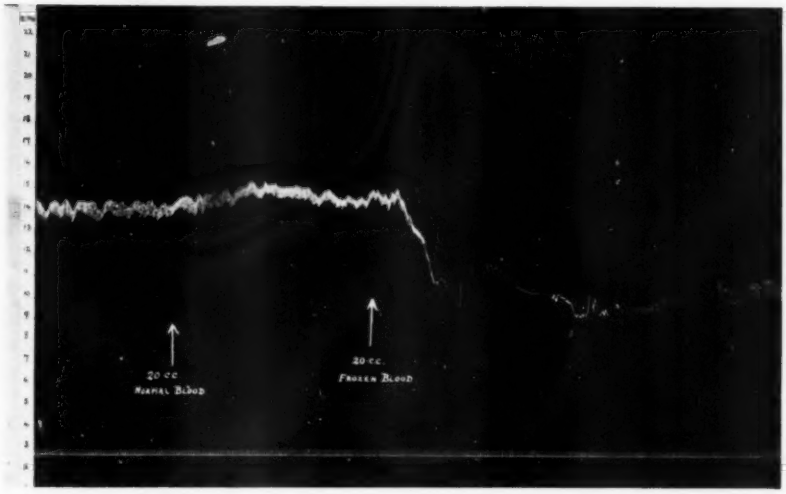

Fig. 1

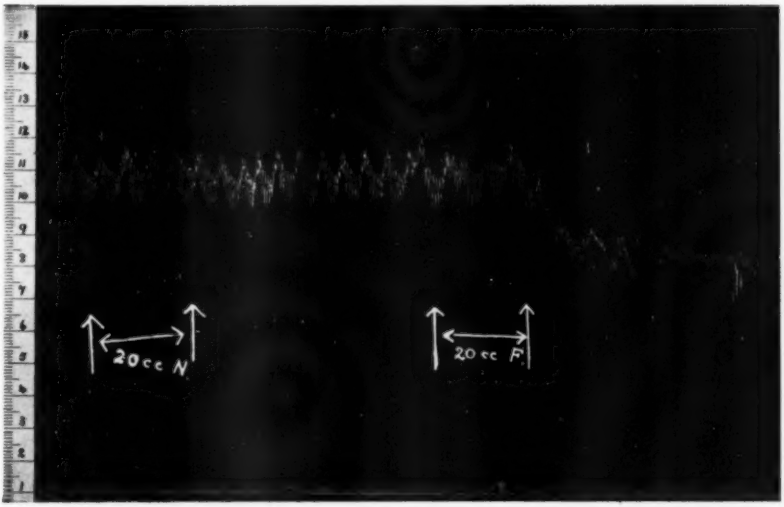

Fig. 2

at once apparent. Serum thus centrifuged and optically pure displayed the same toxicity when injected.

Serum from unfrozen blood was then frozen and thawed, but no toxicity developed.[2]

Blood rendered toxic by freezing was brought to a temperature just under boiling point, to coagulate the proteins. So firm a clot was formed that all attempts to squeeze out a watery solution were unsuccessful. If the clot was rubbed up with a minimum of 0·9% NaCl in a mortar, and then filtered, the filtrate showed no toxic action.

As in so many cases only one injection could be made, an attempt to compare the toxic actions of different dilutions was difficult. This fact, however, came clearly out of the experiments, that when the frozen blood was diluted with three times its volume of 0·9% NaCl, and the whole injected, the toxicity was almost zero.

Another experience at one time promised to throw light on the nature of the toxic agent. In several cases where there was recovery after a first dose, a second dose gave no effect. But in the last experiment I carried out the second injection gave a syncope every bit as pronounced as the first, and indeed killed the animal.

On the whole the evidence points to some protein compound of the corpuscle, liberated and, it may be, denatured by freezing and thawing, as the agent concerned.

CONCLUSION.

Dog's defibrinated blood, itself not toxic, becomes toxic to the donor when frozen and thawed and injected intravenously. This toxicity is not due to embolism from corpuscle "ghosts," but is probably concerned with a protein of corpuscular origin.

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[2] In the course of this investigation another curious fact was observed which has not, I think, been described before. If fresh blood is diluted with twice its volume of distilled water it is haemolysed and no longer reflects light in the same way as does the unhaemolysed. If to a portion of this diluted blood solid sodium chloride is added to bring the concentration of this salt up to 0·9% the diluted blood has the appearance as if its corpuscles had been reconstituted; it becomes redder and reflects light. This change is at present the subject of research in my laboratory.

THE ACTION ON MAMMALIAN CIRCULATION OF THE SKIN SECRETION OF HYLA AUREA

PART I

by

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(Submitted for publication 11th November, 1930.)

The skin secretion of various species of frogs and toads has been investigated from time to time, and substances with digitalis action have been described.[1] That adrenalin can be present has been asserted, but this supposition has been based on the behaviour of arteriolar muscle, and not on chemical properties. That frogs of the genus *Hyla* have skin glands similar in histological features to those in *Rana* has been noted, but I am unaware of any experimentation with the product.

The present investigation began with the experience of having some frog's skin secretion accidentally coming in contact with my conjunctiva. There followed severe pain, especially about the inner canthus. What struck me as remarkable at the time was the fact that though the pain was acute there was no dilatation of the conjunctival vessels. This seemed at first to be an important piece of evidence bearing on the problem of local vaso-dilatation following nociceptive nerve stimulation, but I may at once state that I now believe the absence of reddening to be due to a vaso-constrictor agent present in the secretion. I repeated the eye experiment several times, and always with the same result. On needling some of the surface moisture of the frog into the skin of my arm I perceived in a few minutes a wheal very like that given by histamine, and, what I did not expect after the eye experiments, some reddening.

The experiments now described deal with the action on the circulation of the dog of intra-venous injection of physiological saline containing minute amounts of *Hyla* skin secretion.

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[1] See for example *Digitalis and its Allies*, by A. R. Cushny, 1895; also the chapter, "Tierische Gifte und ihre Wirkung," by Ferdinand Flury in the *Handbuch der Normalen und Pathologischen Physiologie.*

METHODS.

A frog *(Hyla aurea)* was pithed, and its body moved to and fro in a beaker of 0·9% NaCl at room temperature. The toxic substances are so potent that, as a rule, the dog died within a few minutes after injection of a few cc. of this solution. Anything like a quantitative estimation of the active ingredients or a standardized preparation I found impossible. Dogs were morphinized and then anaesthetized with chloroform. The carotid pressure was recorded in the usual way. When a tracing of the heart's action was desired the dog was curarized and artificial respiration was employed; hooks were stuck into the right auricular appendage and into the ventricular apex, and were attached each to a recording lever. In some cases the vagi were cut, in other cases left intact.

RESULTS.

When the intra-venous injection of 5 cc. of the saline skin extract was lethal, and this was the rule, there were three obvious effects: respiration ceased, the heart stopped in systole, and the systemic arterioles went into spasm. I have never seen any drug produce so remarkable an effect on the arterioles, for though the heart was completely out of action the blood pressure remained high for many minutes, and fell only slowly to zero. Actually the arteriolar spasm was so great and so sustained that leakage of blood from the arteries into the capillaries was for the time stopped. At first I thought my carotid cannula had been blocked by a clot, but this possibility was soon set aside. Figure 1 shows these effects clearly: the cessation of heart action, the transformation of normal respiration to occasional gasps, and the slow fall of the arterial pressure (the zero pressure is here on a level with the writing).

When the saline extract was boiled there was some coagulation and the toxicity was reduced. This could, however, be due to an adsorption effect, for several minutes' further boiling did not appreciably lessen the poisonous action.

If the frog's body were moved through the beaker of saline for a short time, or if the extract usually made were diluted with more saline, an effect like that given by adrenalin, or better ephedrine, was obtained. Figure 2 shows the action of both boiled and unboiled preparations; here the vagi were cut, and artificial respiration was used. The sharp rise of pressure is due to peripheral arteriolar stimulation, as was proved by hind limb plethysmography after sciatic section. On the heart a direct stimulation was obvious when sufficiently weak preparations were employed. (Figure 3). This resemblance to adrenalin is certainly striking, but the skin extract gave no true adrenalin reactions; for example there was no darkening on letting the extract stand some days at the ordinary reaction, nor immediate darkening when made alkaline and exposed to the air. Moreover, the arteriolar contraction was gener-

ally more prolonged than that given by adrenalin, and rather suggested ephedrine than adrenalin.

As has been stated, the heart in the majority of cases ceased beating, and a quick post-mortem examination showed this organ to be strongly contracted.

CONCLUSION.

Hyla aurea resembles other frogs in having a skin secretion containing a very powerful principle (or principles) which can act on the heart like digitalis and on the arterioles like ephedrine.

I wish to acknowledge the help I have received in this research from Miss Blanche Brewster, B.Sc.

Fig. 1

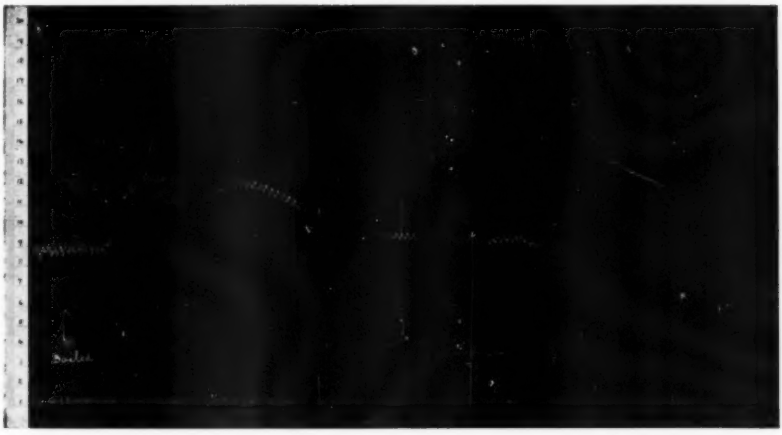

Fig. 2

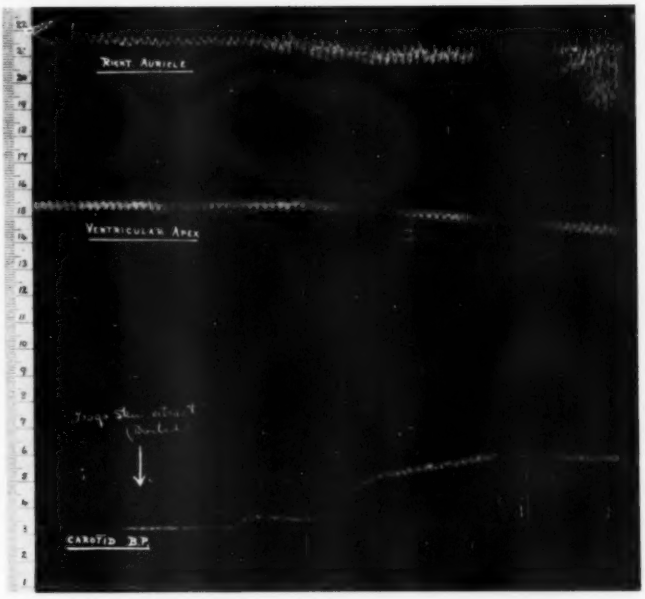

Fig. 3